Genetic Factors Associated with Anti-Factor H Autoantibodies in Atypical Hemolytic Uremic Syndrome (aHUS)

Thesis

GENETIC FACTORS ASSOCIATED WITH ANTI-FACTOR H AUTOANTIBODIES IN ATYPICAL HEMOLYTIC UREMIC SYNDROME

Thesis submitted by

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Abstract

Atypical hemolytic uremic syndrome (aHUS) is a rare form of thrombotic microangiopathy characterized by renal failure and determined by genetic and acquired defects of alternative pathway (AP) of the complement system. Autoantibodies against factor H (anti-FHs), a regulator of the AP, were reported in 10% of patients, and are associated with the deficiency of factor H related 1 (FHR1), a FH homologous protein.

The aim of this thesis was to evaluate the contribution of genetics to the development of anti-FHs in aHUS.

Thirty patients affected by aHUS resulted positive for anti-FHs (9.8%) and FHR1 deficiency was present in 83.3% of them. A healthy control also showed anti-FHs in concomitance with FHR1 deficiency documenting that the lack of FHR1 strongly predisposed to anti-FH development also in healthy subjects although this condition was not sufficient for the disease manifestation.

The presence of infectious prodromal signs and an age at the disease onset around 8 years indicated that common infections may trigger the development of autoantibodies in subjects with at risk genetic background.

Likely pathogenetic variants in complement genes were observed in 37% of our patients with anti-FHs. At variance, common variants in complement genes did not seem to contribute to the disease, as documented by comparing patients with super controls, unaffected subjects carrying FHR1deficiency.

Finally, I report that the *HLA-DRB1*11:04* allele could be a predisposing genetic variant for anti-FH associated aHUS. Further works will be necessary to confirm this finding and to explore the presence of other genetic susceptibility factors that, in combination with the *HLA-DRB1*11:04* allele and the FHR1 deficiency, could increase the risk for anti-FHs.

To Paraskevas and Lydia

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Contribution to the Thesis by other Researchers

Part of the thesis project was carried on with the collaboration of other researchers that contributed to the research as follows:

Internal collaborations in the IRCCS Istituto di Ricerche Farmacologiche Mario Negri

Clinical and biological data of patients were collected through International Registry of HUS/TTP (Ranica, BG, Italy) by Dr. Bresin.

The genetic sequencing of *CFH, CFI, CD46, C3*, *CFB*, and *THBD* by Sanger method was performed by the candidate together with Dr. Rossella Piras, and Mrs Marta Alberti.

Multiplex PCR to evaluate the presence of homozygous *CFHR1* deletion was performed by the candidate together with Dr. Paraskevas Iatropoulos, and Mrs Marta Alberti.

External collaborations

HLA typing was performed by the Unit of Immunogenetics and Transplant Biology directed by Prof. Amoroso (Azienda Ospedaliera – Universitaria, Città della Salute e della Scienza, Torino, Italy) in 33 healthy controls.

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Abbreviations

aHUS	atypical hemolytic uremic syndrome
AIRE	autoimmune regulator
ALCAM	activated leukocyte cell adhesion molecule
ALPS	autoimmune lymphoproliferative syndrome
AMD	age-related macular degeneration
ANOVA	analysis of variance
anti-FH	anti factor H antibody
AP	alternative pathway
APCs	antigen presenting cells
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS1	autoimmune polyendromic pyndrome type 1
AS	ankylosing spondylitis
AU	arbitrary units
B	Benign
BCR	B cell receptor
Bim	bcl-2-like protein 11
C1 INH	C1 inhibitor
C3aR	C3a receptor
C3G	C3 glomerulopathy
C3GN	C3 glomerulonephritis
C3NeF	C3 nephritic factor
C4BP	C4 binding protein
C5aR	C5a receptor
CD	cluster of differentiation
CFH	complement factor H (gene)
CFHR	factor H related (gene)
CNV	copy number variation
CP	classical pathway
CR	complement receptor
CRP	C reactive protein
CTLA-4	cytotoxic T-lymphocyte antigen 4
CXCR	chemokine receptors
DAF	deacy accelerating factor
DAG	diacylglycerols
DDD	dense deposit disease
ddH_2O	double distilled water

DEAP	deficiency of FHRR plasma protein and autoantibody positive form of Hemolytic uremic syndrome
DGKE	diacylglycerol kinase-epsilon kinase
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGR-1	early growth response protein 1
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
et al.	*et alia*
F	female
FB	factor B
FD	factor D
FH	factor H
FHL1	factor H like 1
FHR	factor H related
FI	factor I
FIIa	factor IIa
FIXa	factor Ixa
FXa	factor Xa
FXIa	factor Xia
FXIIa	factor XIIa
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gb3	globotriaosylceramide 3
GPI	glycophosphatidylinositol
GWAS	genome wide association study
HGVS	human genome variation society
HLA	human leukocyte antigen
Hom	homozygous
HRP	horseradish peroxidase
HUS	hemolytic uremic syndrome
i.e.	*id est*
IBD	inflammatory bowel disease
IC	immune complex
iC3b	inactive C3b
IC50	inhibitory concentration
I-CAM	intracellular adhesion molecule 1
IFN	Interferon
Ig	immunoglobulin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X linked

KCNT2	potassium channel subfamily T member 2
LD	linkage disequilibrium
LOD	logarithm of odds (score)
LP	lectin pathway
M	Male
MAC	membrane attack complex
MAF	minor allele frequency
MASP	MBL associated serine proteases
MBL	mannose binding lectin
MCL-1	Induced myeloid leukemia cell differentiation protein
MCP	membrane cofactor protein
MHC	major histocompatibility complex
MLPA	multiplex-ligation dependent probe amplification
MMACHC	methylmalonic aciduria and homocystinuria type C protein
mPCR	multiplex PCR
MPGN	membranoproliferative glomerulonephritis
MS	multiple sclerosis
mTCC	membrane terminal complement complex
mTCEs	medullary thymic epithelial cells
na	not available
nd	not detected
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NK	natural killer
NOD2	nucleotide-binding oligomerization domain-containing protein 2
OD	optical density
OKT3	muromonab-CD3
OR	odds ratio
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PD-L1/2	programmed death ligand 1/2
PECAM-1	platelet endothelial cell adhesion molecule
PIGA	phosphatidylinositol N-acetylglucosaminyltransferase subunit A
PK	protein kinase
PLG	plasminogen
PNH	paroxysmal nocturnal hemoglobinuria
Prob D	probabily damaging
pTEFb	positive transcription elongation factor
PTPN22	protein tyrosine phosphatase, non-receptor type 22
PVDF	polyvinylidene difluoride membrane

RA	rheumatoid arthritis
RAG	recombination activating gene
RCA	regulators of complement activation
Ref	reference
Rem	remission
SCR	short consensus region
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRT1	sirtuin 1
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SSO	sequence specific oligonucleotide probes
SSP	sequencing based typing
SSP	sequence specific primer
STEC	shiga-toxin Escherichia coli
Stx	shiga toxin
TAFI	thrombin-activatable fibrinolysis inhibitor
TCC	terminal complement complex
TCR	T cell receptor
TE	tris-EDTA
TGF	transforming growth factor
THBD	thrombomodulin
TMA	thrombotic microangiopathy
TMB	tetramethyl benzidine
TNFRSF1A	tumor necrosis factor receptor superfamily member 1A
TOP2A	DNA topoisomerase 2-alpha
Treg	regulatory T cell
TSA	tissue specific antigen
TTP	thrombotic thrombocytopenic purpura
VCAM	vascular cell adhesion protein 1
Δ	deletion

1 Introduction

1.1 Complement system

The complement system represents a main part of the innate immune system and it plays an important role to defense the organism from pathogens and to maintain homeostasis. Upon infection complement is activated leading to inflammation, to recruitment of immune cells which are involved in the elimination of the pathogens and in the clearance of immune complexes and cell debris, and to the activation of adaptive immunity.[1-4]

The complement system is composed by more than 30 proteins indicated by the letter C followed by a progressive number, which are either produced by the liver and secreted in the circulation or associated to cell membranes. Complement proteins are zymogens activated by proteolytic cleavages. There are three major pathways of complement activation: the alternative, the classical, and the lectin pathways (Figure 1-1). Each pathway leads to the cleavage of C3 resulting in the formation of activated products and the disruption of pathogens.[1, 2]

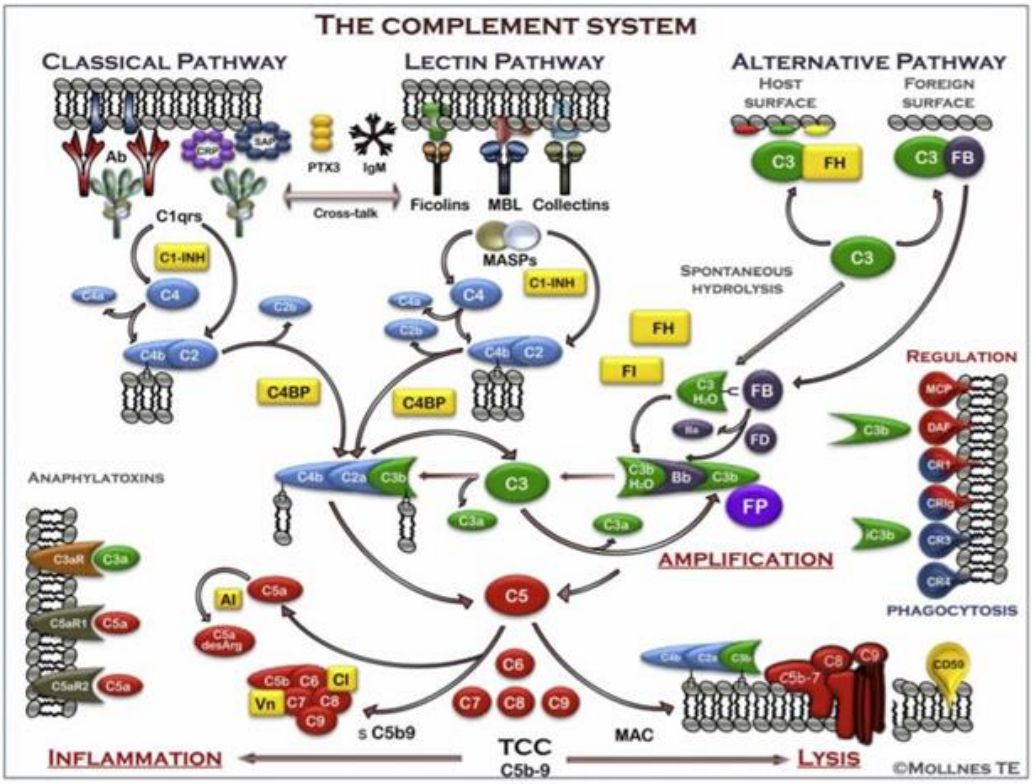

Figure 1-1. Graphical representation of the three pathways of activation of the complement system.

Complement activators and regulators involved in the classical, the lectin, and the alternative pathways are shown (adapted from Ricklin *et al.* Molecular Immunology, 2017).[5]

The alternative pathway (AP) represents the oldest component of the innate immune system and the first components have been identified in a coral suggesting their presence since cnidaria.[6, 7] C3 is continuously hydrolyzed at low rate to generate C3(H_2O) in a spontaneous process called "tick over". C3(H_2O) dramatically changes its conformation and C3 thioester domain flips out exposing the previously hidden thioster bond which recognizes the plasma protein factor B (FB). FB bound to hydrolyzed C3(H_2O), is cleft by the plasma serine protease, factor D (FD) to generate Ba and Bb fragments. Bb remains bound to C3(H_2O) and forms the fluid phase AP C3 convertase which cleaves C3 molecules to C3a

and C3b. Through the tick over process, C3b is constantly produced at low rate in physiological conditions and can bind –OH groups present on pathogens or on other "activating surfaces" which support complement deposition. FB is then recruited to the bound C3b and this association is promoted by factor P (properdin) which is released by activated neutrophils. FD cleaves FB and Bb remains bound to C3b forming the C3bBb AP C3 convertase. Then, C3bBb convertase cleaves other C3 molecules producing an amplification loop and other C3b molecules that remain attached to the cell surface. The other C3a fragment released by C3 convertase activity acts as anaphylatoxin.[1, 2]

The classical pathway (CP) is initiated by binding of the complement protein C1 to immunoglobulins (Igs) present on pathogens or other foreign antigens. C1 is a large multimeric protein complex composed of C1q, C1r, and C1s molecules that binds to the Fc portion of IgG or IgM. C1q subunit is constituted by 6 chains forming an umbrella-like structure with a globular head. C1r and C1s are serine proteases that form a tetramer containing two molecules of each protein. Binding of two or more C1q molecules to IgG or IgM lead to enzymatic activation of the associated C1r, which cleaves and activates C1s. Activated C1s cleaves C4 into C4a and C4b, and C2 into C2a and C2b. C4b contains a thioester bond that forms covalent linkages with Igs. C2b is soluble while C2a remains associated to C4b on the cell surface. The resulting C4bC2a complex is defined as the CP C3 convertase and is able to bind and cleaves C3 molecules. Of note, pentraxin, which belongs to a class of pattern recognition receptors, can interact with pathogens, binds directly C1q, and activates the CP.[1, 2]

The lectin pathway (LP) is activated in the absence of antibodies when either mannose binding lectin (MBL) or ficolins, which are soluble plasma proteins structurally similar to C1q, bind to microbial polysaccharides. MBL is a member of collectin family with a hexameric structure and presents an N-terminal collagen-like domain important for oligomerization and a C-terminal lectin domain which binds the mannose residues on microbial glycoproteins and glycolipids. Ficolins are structurally similar to MBL and show a collagen-like domain and, instead of the lectin domain, present a C-terminal fibrinogen-like domain which binds N-acetylglucosamine and lipoteichoic acid present in the cell walls of Gram positive bacteria. MBL and ficolins circulate associated to MBL-associated serine proteases (MASPs), which are structurally homologous to C1r and C1s proteases, called MASP1, MASP2, and MASP3. Binding to pathogens induces conformational changes leading to auto-activation of MASP2 which is the only MASP that can cleave C4 to form C4a and C4b. C4b recognizes microbial surfaces inducing the binding of C2, which is cleaved by MASP2 to form C2b and C2a. C4b together with the attached C2a forms the LP C3 convertase, C4bC2a, which is identical to the CP C3 convertase.[1, 2] The function of MASP1 and MASP3 are more unclear than MASP2. Of note, MASP-1 seems able to cleave C4b-bound C2 and both MASP1 and MASP3 show the ability to convert the pro-enzyme FD to its active form, suggesting their involvement in both the LP and AP of complement activation. [1, 2, 8, 9]

AP C3 convertases, C3bBb, and CP and LP C3 convertases, C4bC2a, cleave C3 to form C3a and C3b. C3b can bind C3 convertases to form the C5 convertases of the AP, $C3b_2Bb$ and of the CP/LP, C4bC2aC3b. The C5 convertases cleave C5

into the C5a anaphylatoxin and C5b, which remains bound to the cell surface. C5b can bind the terminal complement components, C6 and C7. C7 is hydrophobic and C5b-7 complex is able to insert into the lipid bilayer of cell membranes where it can bind C8 molecules. The formation of terminal complement complex (TCC), C5b9, requires the presence of polymerized C9, the final component of complement cascade. The resulting membrane attack complex (MAC) is able to distort the lipid bilayer of cell membrane leading to the activation of signaling pathways which have an important role in the MAC derived cell lysis..[1, 2, 10] Interestingly, C5b-7 also has the ability to bind the terminal components C8 and C9 forming a soluble complex, called sC5b9, containing the S protein (a plasma glycoprotein produced by endothelial cells, also called vitronectin), and clusterin (also called SP-40). The sC5b9 does not determine the cell lysis but it can bind endothelial cells forming the membrane TCC (mTCC) which promotes the expression of adhesion molecules, inflammation, and increased vascular permeability.[11]

Interestingly, other non-complement proteases are reported to be able to generate complement active products. Coagulation factor XIIa (FXIIa) is able to cleave C1r and thereby promotes the CP activation. Furthermore, thrombin (also called FIIa), FXa, FXIa, FIXa, and plasmin can activate both C5 and C3 and the derived products C3a and C5a are biologically active.[12, 13] Of note, the concentrations of the coagulation factors required to cleave complement proteins in *in vitro* studies are higher than the concentrations present in physiological conditions suggesting that the coagulation components may have a minor effect on complement activation in *in vivo* situation.[13] Indeed, discordant results have been recently

obtained in baboons infused with FXa and phospholipids which led to a strong thrombin and plasmin generation but did not produce measurable levels of complement activation fragments compared to results obtained with infusion with *E. coli*.[14] Finally, FXIIa is able to cleave pre-kallikrein forming kallikrein, which is a serine protease involved in the kinin and plasmin activation, also able to cleave C3 and FB leading alone to the generation of active components of the C3 convertase.[15, 16]

The MAC is one of the main effector of complement activation. Nevertheless, some Gram positive bacteria are able to repair membrane lesions produced by MAC and are resistant to its mediated lysis due to the presence of a very thick cell wall. Another important effector function mediated by complement in the first line of defence against infections is the opsonization of pathogens mediated by C3b and its degradation products iC3b (inactive C3b) and C3d, called opsonins, which lead to their recognition, ingestion, and elimination by phagocytes. The pro-inflammatory effect mediated by opsonins is determined by their interactions with the complement receptors expressed on phagocyte surface (CR1, CR2, CR3, CR4, and CRIg). CR1 is expressed on monocytes, macrophages, neutrophils, erythrocytes, and kidney glomerular podocytes and it binds C3b and C4b. The binding of C3b to CR1 is not sufficient to determine phagocytosis, but enhances IgG mediated phagocytosis of targets recognized by both IgG and C3b molecules. CR3 and CR4 are expressed on macrophages, monocytes, dendritic cells, neutrophils, natural killer (NK) cells, and endothelial cells and are specific receptors for iC3b. CR3 and CR4 belong to integrin family and CR3 presents a lectin site capable of recognizing pathogen carbohydrates. Some tissue resident

macrophages (such as Kupffer cells in liver) express CRIg, a receptor belonging to the Ig family which determines the phagocytosis mediated by iC3b-coated pathogens. Instead, CR2 is expressed on B cells and interacts with C3d and iC3b on surface antigens. The interaction between CR2 and C3d-opsonized antigen induces an increase of B cell receptor (BCR) signaling.[17]

Also the anaphylotoxins C3a and C5a play an important role by enhancing inflammation and by activating immune cells that express anaphylatoxin receptors (C3aR and C5aRs) on their surface. Anaphylatoxins act as potent chemoattractants for neutrophils to sites of inflammation, promoting cell migration and adhesion to the endothelium, trigger oxidative bursts from neutrophils, macrophages, eosinophils, and bind mast cells and basophils inducing degranulation with release of vasoactive mediators such as histamine.[17]

C3aR and C5aRs are also expressed on endothelial cells and their stimulation can lead to a rapid mobilization of Weibel-palede bodies containing von-Willebrand factor, which mediates platelet adhesion, and P-selectin, which promotes the adhesion of leukocytes.[17]

The complement system plays an important role not only in innate immunity, but also in adaptive immunity.

Complement modulates T lymphocyte survival and immune responses acting directly on complement receptors C3aR and C5aR. C3a and C5a can enhance T cell proliferation and inhibit apoptosis, and can increase the strength of the immune response.[17] On the other hand, complement can indirectly enhance cytokine production from antigen presenting cells (APCs) that lead to up-

regulation of costimulatory molecules, amplification the immunological response and determination in which effector phenotype the T cell will differentiate.[17, 18] The complement regulator CD46 (also called membrane cofactor protein, MCP) acts as a costimulatory molecule in CD4+ T cells favoring their activation and proliferation. CD46 is involved in the regulation of T cells ability to respond to interleukin 2 (IL-2) because it is necessary for the normal expression of IL-2 receptors. In addition, in *in vitro* and *in vivo* experiments, T cells isolated from rare CD46-deficient patients are unable to produce interferon and this can determine a susceptibility state for infection diseases.[17, 19]

Complement also plays an important role in B lymphocyte immune responses. As described above, CR2 is expressed on B cell surface and interacts with C3d and iC3b opsonized antigens. The C3d-CR2 interaction leads to an increase of BCR signaling inducing selection and expansion processes of B cells that determine the correct antibody repertoire formation. The C4 binding protein (C4BP) is another protein important in B cell activation because it can bind directly the CD40 costimulatory protein on B cells determining their proliferation and differentiation [20]. Finally, C4 is essential to maintain peripheral B cell tolerance and its deficiency can promote the generation of self-reactive B cells in the germinal centers.[17]

The role of the complement system is not limited to fighting pathogens and promoting inflammation, but it is crucial to promote the clearance of apoptotic cells and immune complexes maintaining homeostasis. During the removal of cellular debris the complement system limits the production of a strong inflammation and of a damage signal. Apoptotic cells express on their surface

signal molecules which allow the opsonization by C3b and C4b and subsequent recognition by phagocytes. However, a number of complement membrane bound or soluble regulators are able to modulate the complement amplification, limiting the complement activation and the inflammation. The CP plays a crucial role in the clearance of apoptotic cells. C1q can directly bind apoptotic cells inducing opsonization by C3b and C4b or indirectly interacting with several molecules present on cell surfaces (such as IgM, pentraxin, C reactive eprotein - CRP -, phosphatidylserine, glyceraldehydes 3-phosphate dehydrogenase – GAPDH -, double strand DNA, and calreticulin). C1q is able to increase the secretion of anti-inflammatory ILs by macrophages, decreasing their expression of CD40, and increasing the expression of inhibitory PD-L1 and PD-L2 (programmed death-ligands) molecules on dendritic cells and macrophages[21]. In general C1q is able to inhibit the inflammasome activity and facilitates the clearance of apoptotic cells avoiding the release of intracellular components which can determine the activation of an immunologic response and the risk to develop autoimmune diseases [21, 22]. Inactive C3b (iC3b) also supports the tolerogenic clearance of apoptotic cells. The opsonization mediated by iC3b and the CR3-dependent phagocytosis determine a downregulation of IL-12, a reduction of oxidative burst in macrophages and of the costimulatory molecule expression and impaired maturation of dendritic cells.[17, 22-24]

1.2 Regulation of complement activation

The activation of the complement system is finely regulated so that it is restricted to microbial cells and foreign surfaces and spares host cells (Figure 1-1).[25]

Complement is also tightly controlled on microbial surfaces to ensure that its activation is limited to the pathogens and is not spread to adjacent normal cells which can be damaged.[25] Regulation is mediated by several circulating and cell membrane proteins that appeared early during evolution.[25] Indeed, regulators of the complement system have been first recognized in Agnatha.[26]

The C3b and C4b deposited on host cell surfaces can be bound by several regulatory proteins, such as CD46, CR1, and factor H (FH), which are not produced by microbes and therefore inhibit the activation of the complement system selectively on host cell surfaces. CD46 is expressed on all nucleated cells, CR1 is expressed on leukocytes, erythrocytes, and glomerular podocytes, while FH is a plasma protein. The presence of high levels of sialic acid on host cells can favor the binding of FH on cell surfaces. By binding C3b and C4b, these regulators compete with the other components of the C3 convertase, such as Bb of AP and C2a of CP, preventing the assembly of the C3 convertases and blocking the progression of the complement activation.

Complement activation is regulated not only by preventing the assembly of the C3 convertase but also by promoting its dissociation. The decay of AP C3 convertase is mainly mediated by FH. CR1, and the decay acceleration factor (DAF/CD55) can mediated the decay of the C3 and C5 convertases of the AP and CP, while C4BP is the major regulator of the CP and the LP.

C3b and C4b are also quickly inactivated by the plasma serine protease FI, which needs the presence of specific cofactors such as CD46, CR1, and FH. FI-mediated proteolytic cleavage generates fragments called iC3b, C3dg, C3c, C4d which are recognized by receptors on phagocytes and B lymphocytes.

The C1 inhibitor (C1 INH) can regulate the CP and the LP. It is a serine protease inhibitor that mimics the normal substrates of C1r and C1s and dissociates them from C1q. Moreover, it also inactivates MASP-1 and MASP-2 proteases in MBL complexes of the LP and inhibits plasma FXII, FXI, and kallikrein.[27, 28]

The terminal complement pathway is also finely regulated. The MAC assembly on cell surfaces is inhibited by S protein, clusterin, lipoproteins, anti-thrombin III, proteoglycans, and protamine, which can bind to nascent C5b-7 complex and prevent its insertion into the cell membrane. Moreover, vitronectin and clusterin interact with the nascent lypophilic C5b-9 making it more soluble and lytically inactive. Protectin, also called CD59, is associated to host cell membrane via a glycophosphatidylinositol (GPI) anchor and by interacting with C9 molecules can impair its polymerization and MAC membrane insertion.[29]

Instead, the inflammation generated by anaphylatoxins is quickly reduced by plasma carboxypeptidases that cleave the C-terminal Arginine, resulting in C3a des-Arg and C5a des-Arg, each of which has less than 10% of their original biological activity.[2] Recent studies have reported that thrombin-activatable fibrinolysis inhibitor (TAFI) can also inactivate C3a and C5a anaphylatoxins.[30] Thrombomodulin (THBD), a transmembrane endothelium glycoprotein, by binding to thrombin enhances the specificity of the latter for TAFI increasing its proteolytoic activation. Moreover, THBD binds C3b accelerating its FI-mediated inactivation in the presence of FH.[31]

1.3 Factor H

FH is the main regulator of the AP of complement system. It has old origin and a homologous FH protein called "sand bass cofactor protein 1 (SBP1) was first described in teleosts.[32]

It is a plasma glycoprotein encoded by the *CFH* gene that is located on chromosome 1q32, in the Regulators of Complement Activation (RCA) gene cluster that includes genes with conserved structures and functions.[33] It is produced mainly by the liver, but is also secreted by endothelial cells, monocytes, fibroblasts, keratinocytes, platelets, and retinal pigment epithelial cells. The plasma concentration of FH is approximately 1-2 μM (about 110 to 615 mg/L) and can reach higher concentrations when it is released locally in tissues.[33, 34]

FH is composed by a single chain constituted by 1213 amino acids and it is formed by 20 short consensus repeats (SCRs) each composed by about 60 amino acids and stabilized by two internal disulfide bonds. Each SCR presents a beta-strand rich domain and a globular conformation. FH has a bent back structure, with a hairpin like form, in which the C-terminus interacts with the N-terminal domains of the molecule.[33] It has eight N-glycosylation sites on SCRs 9, 12-14, 15 (two), 17, and 18, for a total mass of 155 kDa.[33]

FH regulates AP competing with FB for the binding to C3b, inhibiting the assembly of the C3 and C5 convertases, and promoting convertase decay. It also acts as a cofactor for FI in the cleavage and inactivation of C3b.[33, 35, 36]

These regulatory activities are mediated by the four N-terminal SCRs 1-4, which bind with high affinity the C3c part of C3b. SCRs 12-14 and SCRs 19-20 can also bind C3b while SCRs 19-20 are the main binding sites for polyanionic molecules

on cell surfaces (such as glycosaminoglycans and sialic acid). Other binding sites for polyanions have been found in FH SCRs 7-9. Of note, glycosaminoglycans and sialic acids are normally not present on microbial surfaces, so that FH cannot bind resulting in selective complement activation on pathogens.[37]

In addition to C3b and polyanions molecules, FH can also bind CRP, pentraxin-3, fibromodulin, osteoadherin, chondroadherin, prion protein, adrenomedullin, DNA, annexin-II, and histones.[38] The interactions with these molecules allow FH to regulate the complement activation on cell surfaces which are not protected by complement regulators (glomerular basement membrane, extracellular matrix, and apoptotic cells).[38]

CFH encodes for another shorter isoform called factor-H like protein (FHL1) which results from an alternative splicing. It is a 43 kDa protein consisting of the first 7 FH SCRs and 4 additional amino acids (Ser-Phe-Leu-Thr) encoded by *CFH* exon 10. FHL1 has the same N terminal regulatory activities of FH and the SCR7 binding site for polyanions.[33]

The *CFH* promoter controls the expression of both FH and FHL1 which can be modulated by cytokines and inflammatory mediators, such as INF-γ, TNF-α, IL-1, IL-6, and by anti-inflammatory molecules (dexamethasone). Others molecules can influence FH expression such as LPS, retinoic acid and growth factors.[39, 40]

1.4 Factor H related proteins

FH belongs to a protein family that includes five factor H related proteins (FHRs): FHR1, FHR2, FHR3, FHR4, and FHR5. FHRs are produced from the liver and secreted into the circulation. All these proteins are composed by different numbers

of SCR units and show a high similarity suggesting conserved biological functions.[41]

FHRs are encoded by *CFHR1*, *CFHR2*, *CFHR3*, *CFHR4*, and *CFHR5* genes, which are located downstream from *CFH* on chromosome 1q32 within the RCA gene cluster (Figure 1-2). This cluster is characterized by large repeated genomic regions with high degree of sequence identity, which can increase the risk of non-homologous recombination leading to duplication and deletion events. The most common rearrangement reported in *CFHR* genomic region is the polymorphic deletion of 84kb including *CFHR3* and *CFHR1*. The allele frequency of such deletion has high variability across worldwide populations ranging from 0% in Asia to more than 50% in Africa.[42]

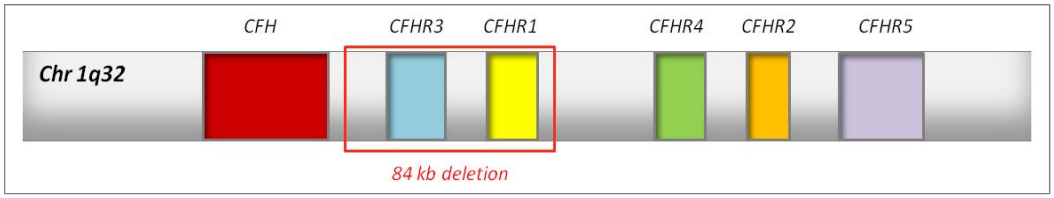

Figure 1-2. Schematic representation of *CFH* and *CFHR* gene cluster on human chromosome 1.

CFH, CFHR1, *CFHR2*, *CFHR3*, *CFHR4*, and *CFHR5* genes are shown. The polymorphic deletion of 84kb including *CFHR3* and *CFHR1* is highlighted in the red box.

FHR proteins are composed by different number of SCRs (Figure 1-3). The N-terminal domains of FHRs are highly homologous to SCRs 6-9 of FH which bind polyanions present on cell surfaces. The amino terminal SCRs 1 and 2 of FHR1, FHR2, and FHR5 are highly conserved and mediate protein dimerization leading to FHR homodimers and heterodimers. On the contrary, FHR3 and FHR4 lack the N-terminal dimerization domain and circulate as monomers. The FHR C-terminal

domains show a very high homology with the FH C-terminus that includes the anchor site to cell surface and the C3b recognition site, suggesting the presence of conserved protein domains that can bind similar surface molecules.[34, 41]

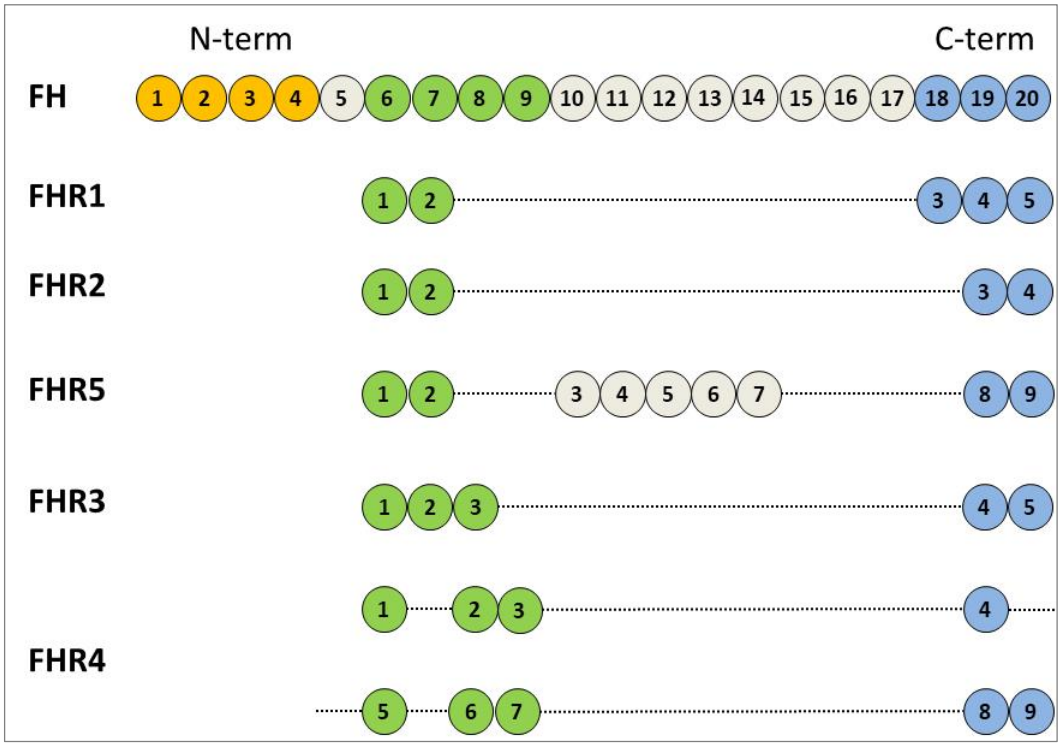

Figure 1-3. The FHR protein family.

Each short consensus region (SCR) of FH and FHRs is indicated by a circle. N-terminal, central, and C-terminal domains of FH, FHR1, FHR2, FHR3, FHR4, and FHR5 are shown. Protein domains with conserved amino acid sequences and functions are shown with the same colors (adapted and modified from Skerka *et al.* Mol Immunol 2013).[41]

For the purpose of this thesis, here I focus on FHR1.

FHR1 has a plasma concentration about 70-100 ug/ml and given that FH has a higher mass than FHR1, the FHR1/FH molar ratio is estimated to be 0.3:1. FHR1 circulates in two different glycosylated isoforms, alpha (37 kDa) and beta (42 kDa). It presents a dimerization domain at N-terminus and can form homodimers and heterodimers with FHR2 and FHR5. It can also be present in the circulation

associated to high-density lipoprotein particles.[34] FHR1 is composed by five SCRs. SCRs 1 and 2 have high degree of amino acid identity with SCRs 1-2 of FHR2 (97% and 100%) and with SCRs 1-2 of FHR5 (91% and 83%). The similarity at N-terminus SCRs 1-2 with the FH SCRs 6-7 is not very high (34% and 42%, respectively) while a high degree of sequence identity is present between C-terminal SCR3-5 of FHR1 and FH SCRs 18-20 (Figure 1-4). Of note, the FHR1 SCR4 and FH SCR19 are identical. There are two different FHR1 isoforms: acidic FHR1 (called, FHR1A, pI = 7.1) characterized by SCR3 157His, 159Leu and 175Glu and basic FHR1 (called FHR1B, pI = 7.53) with SCR3 Tyr157, Val159 and Gln175 which corresponds to FH SCR18 (Tyr1058, Val1060 and Gln1076) and makes SCR3 of FHR1 identical to FH SCR18. Instead, FHR1 SCR5 differs from FH SCR20 for 2 amino acids: Leu290 (that corresponds to Ser1191) and Ala296 (corresponding to Val1197).[34]

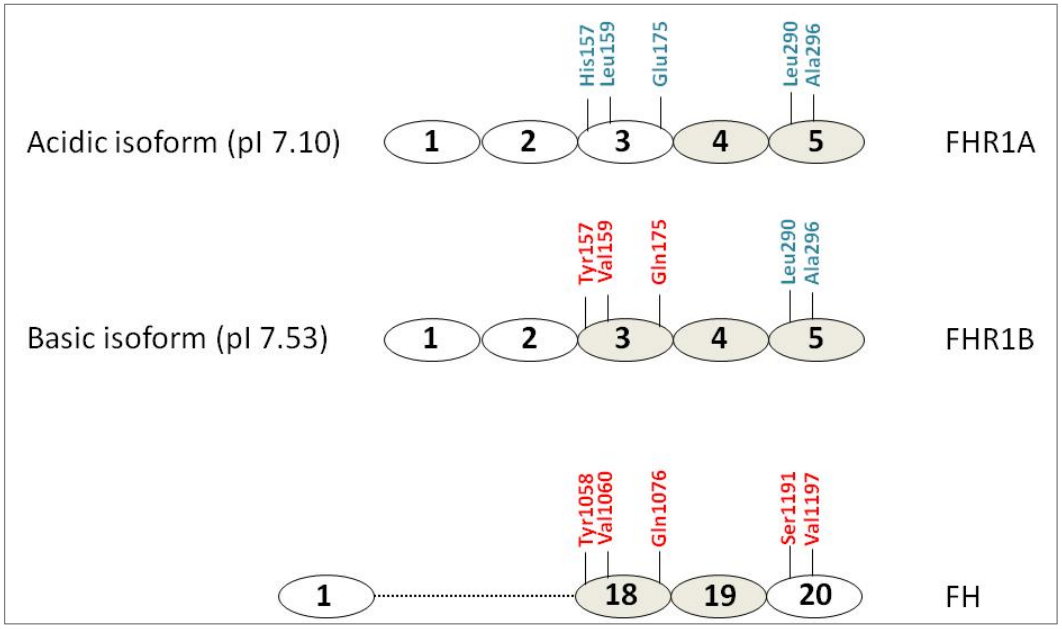

Figure 1-4. Alignment of FHR1A SCRs 3-5, FHR1B SCRs 3-5 and FH SCRs 18-20.

The SCR 3 of FHR1B is identical to the SCR18 of FH as FHR1 Tyr157, Val159, and Gln175 correspond to FH Tyr1058, Val1060, and Gln1076. On the contrary, the SCR5 from both FHR1A and FHR1B is different from FH SCR20 for the presence of Leu290 and Ala296 (adapted and modified from Abarrategui-Garrido *et al.* Blood, 2009).[43]

FHR1 has been reported to bind C3b in the C5 convertase preventing the progression of the complement activation mediated by C5.[44] During the hemolytic assay performed on sheep erythrocytes, it can reduce the generation of C5a but not of C3a, suggesting its inhibitory activity is restricted to C5 convertase.[44] Indeed, the C5b deposition was absent on erythrocyte surfaces in presence of FHR1.[44] Furthermore, it seems that FHR1 can inhibit the assembly of the TCC by binding C5 and C5b6 through its N-terminal domain, but this observation needs confirmation.[44, 45] FHR1 does not show FI cofactor activity and C3 convertase decay activity.[41] It is suggested that FHR1 may compete with FH for the C3b binding through its C-terminal domain promoting C3 convertase activity, although it has lower plasma concentration than FH. Furthermore, dimerization mediated

by N-terminal domain can increase the FHR1 ligand avidity causing an efficient competition with FH for the binding to the C3b. [34, 41, 45]

1.5 Mechanisms of complement evasion by pathogens

The complement system plays a central role in defense against infections but across evolution some pathogens have developed several strategies to escape complement attack and these mechanisms are determinant for their virulence.[46]

The recruitment of soluble complement regulators is a common strategy adopted by several bacteria, but it is also described in viruses, fungi, and parasites. In many microbes, C4BP, FH, and FH-like1 have been found associated to the pathogen cell surface proteins to give protection from complement activation (*i.e.* *Escherichia coli*, *Borrelia* spp, *Neisseria* spp, and *Streptococcus* spp). The FH amino acids localized at SCR20 (Arg1182, Arg1203, and Arg1206) are mainly involved in interaction with pathogens as described for OspE protein from *B. burgdorferi* which can bind FH SCRs 19-20.[47] Other binding sites are also reported in FH SCR6-8. For example, Por1A protein from *N. Gonorrhoaea* can interact with both SCRs 19-20 and SCR6 of FH.[47, 48] Membrane-bound regulators such as MCP, DAF, and CD59 are also suggested to mediate evasion mechanisms although the primary strategies to escape complement attack are represented by the soluble complement regulators.[46] Another evasion mechanism consists in the expression of complement regulations-like proteins. Viruses like orthopoxvirus variola, monkeypox, and cowpox produce RCA-like proteins with typical SCR domain structure which show complement inhibitor activities, like cofactor activity and decay acceleration activity. The degradation of complement

components into non-functional fragments is another evasion strategy. The degradation of C1q is described in *Pseudomonas* spp and it prevents the activation of CP. Furthermore, properdin is degraded by *Streptococcus* spp and inactivation of C3 is reported in *Pseudomanas* spp and *Porphyromonas* spp. Other pathogens directly bind complement proteins leading to inhibition of their activity. *Staphylococcus aureus* through SpA protein recognizes Fc domain of IgG blocking the C1q binding sites. The CD59-like protein from *Borrrelia burgdorferi* and SIC protein from *Streptococcus aureus* inhibit MAC formation by binding its components. In *Herpes* viruses, gC1 and gC2 glycoproteins bind C3b and accelerate the decay of the AP C3 convertase. Other proteins which directly interact with the complement system have been found in parasites, such as *Schistosoma* and *Trypanosoma*. [17, 46]

1.6 Complement mediated diseases

Since the complement system represents one of the most important lines of defence of innate immunity against pathogens, defects in its activation can predispose to infections. Indeed, complement deficiencies have been associated with several infectious diseases. The loss of the opsonization mediated by complement receptors CR3 and CR4 is reported to predispose to pyogenic infections. C2, C3, and C4 deficiency can also predispose to susceptibility for *Neisseria, Haemophilius influenzae* and other respiratory tract infections. Defects in terminal complement pathway that impair the complement lytic activity mediated by complement components from C5 to C9 can also increase the probability of Neisserial infections and the same applies to deficiencies in FB and

FD. Properdin deficiency is also reported to predispose to mortality associated to *Neisseria meningitides* infections. Interestingly, low levels of MBL in young children are associated with recurrent pyogenic infections and failure to thrive. Deficiency of complement receptors is also implicated in human infections. For example, CR1 expressed on erythrocytes acts as a receptor for *Plasmodium falciparum* and a low expression is reported to protect against malaria. [2, 49]

As, the role of the complement system is not limited to promote inflammation to fight infections, but it is also important in the clearance of apoptotic cells and immune complexes, some complement deficiencies predispose to the development of inflammation and autoimmunity. Homozygous deficiency of the first components of the CP (C1q, C1r, and C1s, and more rarely C4, and C2), is associated with an increased susceptibility to systemic lupus erythematosus (SLE). SLE is a systemic autoimmune disease characterized by specific clinical features, such as joint pain, fever, malar rash, fatigue, glomerulonephritis, and hemolytic anemia. The disease is associated with the presence of autoantibodies which appear as the consequence of the immune response induced by an inefficient clearance of apoptotic cells. It was observed that, on specific genetic backgrounds, C1q-deficient mice develop antinuclear antibodies and glomerulonephritis characterized by uncleared apoptotic bodies, suggesting that C1q is crucial to promote the clearance of apoptotic cells preventing the exposure of self-antigens and reducing the risk of autoimmunity.[23, 50]

The complement system is finely regulated to maintain a homeostatic balance. When this equilibrium is perturbed by regulation defects, complement is

continuously activated leading to acute or chronic inflammatory diseases and also autoimmunity.[17]

The deficiency of C1-INH leads to the uncontrolled and spontaneous activation of C1 with the consumption of C4 and C2. Since C1-INH is the most important inhibitor of plasma FXII, FXI, and kallikrein, its deficiency can predispose to hereditary angioedema, a rare disorder characterized by an increased vascular permeability and swelling caused by leakage of fluid from blood vessels into connective tissues.[51]

CD59 and DAF are both GPI-linked molecules. In paroxysmal nocturnal hemoglobinuria (PNH), hematopoietic cell clones in PIGA (phosphatidylinositol N-acetylglucosaminyltransferase subunit A), a protein involved in the synthesis of GPI anchors, have been found. The result is a decreased expression of GPI-linked proteins, mainly CD59, which results in hemolytic anemia caused by complement-mediated lysis of PIGA-deficient red blood cells.[52]

AP dysregulation is well documented in membranoproliferative glomerulonephritis (MPGN), a kidney disorder characterized by proteinuria in nephritic or nephrotic ranges, hematuria, hypertension, and glomerulonephritis. By immunofluorescence microscopy, MPGN has been reclassified in immune complexes mediated MPGN (IC-MPGN) and C3 glomerulopathy (C3G). IC-MPGN is characterized by glomerular C3 staining and significant Ig and C1q deposition. On the contrary, C3G is characterized by dominant C3 deposition with no or scanty Ig and C1q. On the basis of electron microscopy C3G is subdivided into dense deposits disease (DDD) with osmiophilic mesangial and intramembranous dense deposits, and C3 glomerulonephritis (C3GN) with

amorphous mesangial, subendothelial, and subepithelial deposits.[53] Low serum C3 levels associated with auto-antibodies that stabilize the AP C3 convertase (C3NeFs, which means "C3 nephritic factors") are observed in most C3G patients, especially in DDD patients.[54] In rare C3G patients, autoantibodies to factor H, factor B or C3b have been also identified. Interestingly, genetic defects in genes encoding C3, FB, FH, FI and CD46 have been described in both C3G and IC-MPGN patients suggesting that genetic abnormalities in the AP are implicated in the pathogenesis of both diseases.[52, 54, 55]

Genetic and acquired defects of the AP are strongly associated to atypical hemolytic uremic syndrome (aHUS), a rare thrombotic microangiopathy characterized by renal involvement.[52] The details of genetic and acquired drivers in aHUS will be described in the next sections.

Age-related macular degeneration (AMD) is the leading cause of visual disability in individuals over 60 years and is characterized by progressive dysfunction of the retinal pigment epithelium and visual loss. The FH common variant 402 Hys has a great impact on the AMD progression because it affects the FH binding to glycosamminoglycans leading to accelerated protein deposition in Brunch's membrane known as "drusen" which determines the progressive loss of retinal cells. [52, 56]

Involvement of complement are described in other inflammatory and autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), Alzheimer's disease, autoimmune myocarditis, and asthma. Animal models, in which complement proteins are specifically manipulated, have provided strong evidence

of the involvement of the complement system in the pathogenesis of these diseases.[52]

1.7 Hemolytic Uremic Syndrome

The hemolytic uremic syndrome (HUS, also defined as typical HUS) is a rare disease belonging to the group of the thrombotic microangiopathies (TMAs). It is characterized by non-immune microangiopathic hemolytic anemia (Coombs negative), thrombocytopenia, and acute kidney injury. It has an annual incidence of 1-2 cases per 100,000 people and a worldwide prevalence around 1:10,000 (http://www.orphanet-italia.it).[57] HUS occurs mainly in children and has a good prognosis despite in the last outbreak in Germany (2011) many affected adults (88%) had severe manifestations. The typical HUS in children is also called STEC-HUS because it is mostly triggered by strains of Shiga-like toxin (Stx)-producing *Escherichia coli*, such as O157:H7, O111:H8, O104:H4, O103:H2, O145, O123, O121, and O26 serotypes and other Stx-producing bacteria, such as *Shigella disenteriae* serotype 1.

STEC infection can be acquired by humans consuming contaminated food or water and from contact with infected animals or humans (or their excreta). Diagnosis is based on detection of Stx-*E. coli* in stool cultures, anti-Stx or anti-*E. coli* LPS serological tests and molecular tests. After the exposure to the pathogens, about half of cases show hemorrhagic colitis and 3-20% of cases develop HUS. Seventy-percent of patients who progress to HUS need blood transfusions, 50% dialysis, and 25% present neurologic signs. In the developed countries about 5% of patients die during the acute phase of the disease.[58]

There are two Stxs, Stx-1 and Stx-2, which are characterized by different degrees and types of tissue damage. Both Stx-1 and Stx-2 are about 70 kDa proteins and are composed of a single A subunit and five B subunits. After oral ingestion, *E. coli* reaches the gut and adheres to epithelial cells. Stxs are picked up by gastrointestinal epithelium via transcellular pathway and translocate into the circulation, probably bound to circulating polymorphonuclear leukocytes. Through the B subunit, Stx binds the globotriaosylceramide Gb3 receptor which is highly expressed on glomerular endothelial cell surface. Stx-2 is 1000-fold more toxic than Stx-1 on human endothelial cells *in vitro* and this can be explained by its very slowly dissociation rate from Gb3. After internalization by receptor-mediated endocytosis, Stxs are transported through the Golgi apparatus to the endoplasmic reticulum, where the A and B subunits dissociate. The A subunit is translocated to the cytosol where it blocks the protein synthesis, and to the nucleus where it leads to the upregulation of several genes encoding chemokines (IL-8, MCP-1, and SDF-1), chemokine receptors (CXCR4 and CXCR7), cell adhesion molecules (P-selectin, I-CAM, PECAM-1, and VCAM) and transcription factors (EGR-1 and NF-KB), favoring leukocyte recruitment. Stx also induces endothelial apoptosis by the inhibition of the expression of anti-apoptotic *MCL-1* gene. Finally, Stxs can also directly activate platelets and inflammatory cells causing inflammation and microvascular thrombosis.[58, 59]

The histological lesions in patients with STEC-HUS are characterized by thickening of arterioles and capillaries, swelling and detachment of endothelium, accumulation of subendothelial proteins and cellular debris, and presence of thrombi which obstruct endothelial vessels. TMA lesions are typically detected in

glomeruli but also in many other organs, such as the brain, heart, lungs, gastrointestinal tract, and pancreas. Abnormal or fragmented erythrocytes are also a clear evidence of hemolysis in blood smears.[57]

The therapy for STEC-HUS is based on supportive treatments. Early antibiotic therapy rapidly can resolve hematologic and renal abnormalities with the exception of Stx-HUS by *S. dysenteriae* serotype 1 which can lead to death unless antibiotics are started early. In general, the late antibiotic treatments can increase the risk of complications because it might favor the acute release of large amounts of toxins. The anti-C5 humanized monoclonal antibody, Eculizumab, which blocks the terminal pathway of the complement system, was used during the last German STEC-HUS outbreak, alone or in combination with plasma exchange. No significant difference in treatment efficacy has been reported in patients who received the combined therapies compared with patients who received the plasma exchange alone.[58]

Neuroamminidase producing pathogens, such as *Streptococcus pneumonia* have been also reported to be an uncommon cause of HUS. Neuroamminidase also called sialidases, catalyze the hydrolysis of terminal sialic acid residues from cell surfaces determining the exposition of the Thomsen-Friedenreich (T) antigen, which is recognized by IgM leading to erythrocyte agglutination, hemolysis, microvascular thrombosis, thrombocytopenia and the clinical picture of HUS. Early recognition of this form of HUS is critical to avoid therapy based on plasma or blood products because a lot of healthy donors contain anti-T IgM in their serum. The use of washed blood products has been shown to improve the outcome.[60, 61]

1.8 Atypical Hemolytic Uremic Syndrome

Ten percent of HUS cases are not caused by Stx-*E. coli* or *S. pneumonia* infections. These patients are characterized by an uncontrolled activation of the AP of the complement system caused by genetic or acquire defects in AP complement components and regulators and the disease is defined as atypical HUS (aHUS). Atypical HUS is more severe than STEC-HUS, and before the introduction of anti-complement therapy, the clinical outcome was unfavorable: up to 50% of cases progressed to end-stage renal disease (ESRD) and 10-15% died during the acute phase of the disease.[57]

Familial forms are described in less than 20% of cases of aHUS and had a poor prognosis: 50-80% of patients developed end-stage renal disease or died. Both autosomal dominant and recessive inheritance have been reported. Atypical HUS that develops in patients who do not have affected family members is defined as sporadic. [57]

The disease can be preceded by proteinuria in nephrotic range, diarrhea may occurs but it is not a common feature. Different triggers can be associated to the disease manifestation, as bacteria, virus and parasite infections (often upper respiratory tract and non-Stx gastrointestinal infections), cancer, organ transplantation, pregnancy, and anticancer therapies (mitomycin, cisplatin, bleomycin, and gemcitabine), immunotherapeutic agents (cyclosporine, tacrolimus, OKT3, and IFN), and contraceptive drugs. In about 10% of female patients with aHUS, the disorder develops during pregnancy or post-partum.[57]

The presence of inherited defects that determine uncontrolled activation of complement system on host cell surfaces has been documented in familial cases and also in sporadic patients with aHUS. Of note, in about 40% of sporadic cases no genetic defects have been found.[62]

Clinical and histological features

The diagnosis of aHUS is based on the presence of specific clinical features. The most important is the presence of microangiopathic hemolytic anemia which is defined by hematocrit less than 30%, hemoglobin less than 100 g/L, serum lactate dehydrogenase greater than 460 U/L, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, platelets less than 150×10^9/L and negative Coombs test to exclude autoimmune hemolytic anemia. Other important inclusion criteria for the aHUS diagnosis are the presence of acute kidney injury with hematuria, proteinuria and/or reduced renal function, the absence of STEC infections to exclude typical HUS, and ADAMTS13 activity >10% to exclude thrombotic thrombocytopenic purpura (TTP). Extra-renal manifestations are described in about 20% of aHUS patients including seizures, coma, cardiac ischemic events, pulmonary hemorrhage, pancreatitis, hepatic cytolysis, and intestinal bleeding.[57] Patients with aHUS may have reduced serum levels of complement C3 with normal levels of C4, as a consequence of C3 activation and consumption through the AP. High levels of activated complement components, such as C3b, C3c, and C3d are also present.

The kidney histology shows the typical TMA lesions with intima proliferation and thrombosis signs, which are indistinguishable from those of STEC-HUS. Patients

with aHUS and low serum C3 levels show high levels of C3 and C9 deposits in glomeruli and small arterioles during the acute phase of the disease indicating the presence of uncontrolled complement activation in the kidney and a local C3 consumption. The hyperactivation of complement leads to glomerular endothelial cell damage and to the formation of thrombi, which consume platelets and obstruct blood vessels. Erythrocytes are deformed and lysed by passage through microvessels obstructed due to the presence of thrombi, causing hemolytic anemia.[57]

Therapy

Eculizumab (Soliris, Alexion Pharmaceuticals) is a high-affinity humanized anti-C5 monoclonal antibody which binds specifically to C5 preventing its cleavage into C5a and C5b and blocking the generation of MAC. It is administered to patients with paroxysmal nocturnal hemoglobinuria (PNH) because it reduces intravascular hemolysis, hemoglobinuria, and the need of transfusions.[63] Successful therapy with Eculizumab has been reported in literature and clinical trials in more than 80% of aHUS cases.[64-66] The drug is highly effective to treat aHUS on native kidneys and also to prevent post-transplant aHUS recurrences.[67] Eculizumab efficacy has been demonstrated both in patients with or without genetic defects.[65] Of note, Eculizumab increases the risk of meningococcal infections, and vaccination in combination with antibiotic prophylaxis is recommended.[68] Before the introduction of Eculizumab for the therapy of aHUS, plasma exchange was used as the gold standard in management of aHUS patients and it is still used if Eculizumab is not available or in the emergency treatment of critical patients with severe TMA.[68] Plasma treatments reduced the mortality rate

in aHUS patients from 50% to 25%. Plasma exchange appears more effective than plasma infusion because it eliminates toxic molecules and mutant proteins from the circulation.[57] Plasma therapy induced remission also in patients with genetic mutations in complement system. In some patients with severe hypertension and extensive microvascular thrombosis, bilater nephrectomy has been performed to achieve blood pressure control.[57]

Genetics

Dysregulation or hyperactivation of the AP of complement system are the main causes of aHUS. In about 40% of familial cases the complement activation is related to the presence of loss-of-function mutations in the *CFH* but also 10-20% of sporadic cases show *CFH* genetic abnormalities.[62] The large majority of *CFH* mutations are heterozygous point mutations which lead to amino acid changes or to truncated proteins and cluster mainly in the C-terminal part of FH which is a crucial domain for the FH binding to the endothelial cells and to ensure FH mediated surface protection from the complement activation.[69] Since FH can form oligomers through its C-terminus, the presence of heterozygous defects in this domain can lead to a dominant negative effect, caused by the functional blocking of the wild type FH.[70] Anyway, in most cases, the haploinsufficiency is the mechanism that determines the aHUS phenotype, as documented by hemolytic assay in which the lysis of sheep erythrocytes occurs in presence of serum from aHUS patients carrying heterozygous mutations in *CFH* and is stopped addition of normal serum.[71] Homozygous mutations have been found in about 15% of patients and lead to complete FH deficiency and very low C3 levels.

Genomic rearrangements in *CFH-CFHR* cluster have been also described in a few aHUS cases and are favored by the presence of repeated regions with a high degree of sequence identity. *CFH/CFHR1* and *CFH/CFHR3* hybrid gene and *CFHR1/CFH* reverse hybrid genes which encode FH/FHR1, FH/FHR3, and FHR1/FH fusion proteins have been reported in both familial and sporadic cases. Functional studies have demonstrated that *CFH/CFHR1* and *CFH/CFHR3* hybrid genes can determine a complement dysregulation at cell surface since the C-terminal domain of FH is substituted by the C-terminal domain of FHR1 leading to the formation of an abnormal regulator which is less efficient to protect endothelium than FH. Instead, *CFHR1/CFH* reverse hybrid gene was described to be a strong competitor of FH for the binding to the cell surface since during the hemolytic assay addition of purified FHR1 from heterozygous *CFHR1/CFH* hybrid gene carriers determined a dose-dependent sheep erythrocyte hemolysis compared to the addition of equal amounts of purified FHR1 from normal serum which had no effect.[72-76]

Genetic abnormalities have been also found in other regulatory proteins of the AP, such as *CD46* and *CFI*.[62, 77-79] Mutations in *CD46* are present in about 10-15% of aHUS cases and are usually heterozygous mutations, although also homozygous or compound heterozygous mutations have been found. *CD46* mutations usually affect the extracellular domains of the protein which are implicated in the C3b binding and in the cofactor activity for factor I.[80, 81] Mutation in *CFI* are described in 5-10% of patients with aHUS. Only heterozygous mutations in *CFI* are reported in literature and the most of them cluster in the serine protease domain. Half of patients with defects in *CFI* shows low FI plasma levels.[80, 82]

Heterozygous mutations in *CFB* and *C3* are reported in about 2% and 4-10% of aHUS cases, respectively. Some defects in C3 gene can lead to impaired binding of C3b to the regulator MCP and thus resistance to cleavage by FI.[83] Instead, other C3 abnormalities can increase the binding affinity of C3 to FB causing a more stable C3 convertase.[84] Mutants of FB are gain-of-function mutations which lead to an enhanced formation of the C3 convertase that is resistant to the inactivation mediated by complement regulators.[85]

Incomplete penetrance of the disease suggests that additional genetic factor and/or environmental hits are necessary for aHUS manifestation. In about 3% of aHUS patients combined mutations are reported: 25% of patients with mutations in *CD46* and *CFI* also carried defects in other genes while only 8-10% of patients with *CFH*, *C3*, and *CFB* defects also carry other mutations suggesting that these mutations are sufficient alone to predispose to aHUS.[86] Moreover, about half of the individuals carrying *CFH* or *CD46* mutations are healthy subjects suggesting that these mutations could be predisposing for the disease development but need other risk factors for the disease to manifest.[80]

Also common variants in *CFH* (single nucleotide polymorphisms, SNPs) have been described to increase the risk to development of aHUS in subjects carrying mutations in complement genes. *CFH* c.1-332 C>**T** (rs3753394), c.2016 A>**G** p.Gln672Gln (rs3753396), and c.2808 G>**T** p.Glu936Asp (rs1065489) are found to be predisposing factors in aHUS patients.[87] No evidences of functional effects have been reported for these *CFH* variants, but all of them belong to the H3 *CFH* haplotype (CFH_{TGTGGT}), which is defined by c.1-332**T** (rs3753394), c. 184**G** p.Val62 (rs800292), c.1204**T** p.Tyr402 (rs1061170), c.2016**G** p.672Gln

(rs3753396), IVS15 −543**G** intron 15 (rs1410996) and c.2808**T** p.936Asp (rs1065489), and it is strongly associated with aHUS risk.[88] Furthermore, Bernabeu *et al.* observed the presence of an extended *CFH-CFHR3-CFHR1* haplotypes defined by the *CFHR3* SNPs rs446868, rs138675433, and rs138675433 and the *CFHR1* SNPs rs4230, and rs414628 in addition to the *CFH* SNPs above described. The extended *CFH(H3)-CFHR3*B-CFHR1*B* haplotype was found to be strongly associated with aHUS and with lower FH levels.[89] The FH 402Hys variant which has no direct effect on complement activation but can influence the binding of FH to cell surface, has not been found in association with aHUS but it can increase the risk for AMD and DDD, despite conflicting results have been reported in a recent study on DDD in which no significant association between FH 402Hys and the disease was present[55, 90, 91]. Functional studies on FH 62Ile variant have demonstrated that is able to increase the FH ability to bind to C3b which leads to an enhanced cofactor activity and iC3b formation. This variant is associated with a lower risk to develop aHUS, AMD, and DDD.[92, 93]

Esparza-Gordillo *et al.* described a *MCP* haplotype (MCP_{GGAAC}), which is defined by c.1-652**G** (rs2796267), c.1-366**G** (rs2796268), c.1-78**A** (rs1962149), c.1+638**A** (rs859705) and c.1+4070**C** (rs7144), as predisposing to an increased risk of aHUS.[94]

Other common polymorphisms in FB and C3 were reported to influence the risk to develop human immune-mediated diseases despite they have never been described in association with aHUS. The FB 32Gln variant shows a lower affinity for C3b compared to the Arg variant and forms the AP convertase less efficiently. The FB 32Gln is a protective variant for AMD but it is a predisposing genetic

factor in infections and is overrepresented in autoimmunity.[92] The C3 102Gly and 314Leu variants also increase the risk of DDD and are also associated with IgA nephropathy, systemic vasculitis, and kidney allograft dysfunction.[92, 95] Harris *et al.* showed that although each polymorphism can determine only a small change in complement activity, the presence of an inherited set of the more active common variants (defined as "complotype") can contribute to the inflammatory disease phenotype.[96]

Of note, abnormalities in complement genes were reported in about 60% of aHUS cases and also acquired defects, such as antibodies against FH and FI have been described.[97, 98] Others aHUS cases can be determined by genetic drivers not belonging to the complement system. Important pathways that are linked to the complement system are the coagulation and the fibrinolysis. Indeed, coagulation/fibrinolytic proteins such as Thrombin, FXIa, FXa, FIXa, and plasmin can directly cleave C5 and C3 determining the activation of complement independently from other complement components.[13] Alterations in proteins of the coagulation/fibrinolytic pathway, like thrombomodulin (THBD) and plasminogen (PLG) have been reported in aHUS cases.[31, 99] Heterozygous missense mutations in *THBD* are reported in about 5% of aHUS cases and *in vitro* studies have demonstrated that they can determine a moderate reduction of THBD expression on cell surface and an inefficient regulation of the complement AP activation caused by a defect of FI-mediated C3b inactivation.[31] Moreover THBD abnormalities cause impairment in thrombin activatable fibrinolysis inhibitor (TAFI) activation impairing the degradation of C3a and C5a. The THBD common variant Ala473 has been also reported to be associated to an increased risk to

develop C3G.[55] *PLG* mutations are reported to affect about 5% of aHUS patients. Three *PLG* variants which cause plasminogen deficiency have been found in aHUS patients suggesting that a decreased activity of plasminogen/plasmin can predispose to aHUS caused by inefficient thrombi degradation.[99]

A rare recessive form of aHUS is associated with mutation in *DGKE* which encodes the diacylglycerol kinase-epsilon kinase. Arachidonic acid-containing diacylglycerols (DAG) activate protein kinase C, promoting thrombosis and DGKE can inactivate DAG, producing phosphatidic acid which confers protection from thrombi formation. Loss of function mutations in DGKE have been found in aHUS patients with a disease onset before 1 year of age. These mutations lead to the activation of endothelium, complement activation and prothrombotic phenotype.[100, 101]

Cobalamin C deficiency is also another non-complement form of HUS. It is rarer than complement mediated aHUS and it is determined by a recessive disorder of vitamin B12 metabolism. It is caused by mutations in MMACHC gene which are associated to methylmalonic aciduria and homocysteinuria, conditions characterized by elevated levels of methylmalonic acid and homocysteine and decreased methionine.[102]

1.9 Anti-FH antibody mediated aHUS

In 2005 Dragon-Durey *et al.* described the presence of auto-antibodies against FH (anti-FHs) in three children affected by aHUS who presented recurrent disease, identifying for the first time an autoimmune form of aHUS.[98]

Detection of anti-FH antibodies in aHUS patients and in healthy controls

The presence of anti-FH antibodies (anti-FHs) was evaluated by Dragon-Durey *et al.* using an ELISA assay in which 5 μg/mL of purified FH were coated on a plate (Nunc MaxiSorp, Roskilde). After washing and blocking with PBS with 1% BSA, 1:50 diluted patient plasma was incubated for an hour at room temperature. After washing, goat anti-human IgG antibody specific for γ chain and conjugated with horse radish peroxidase (Sigma Aldrich, Steinheim), was incubated for an hour at room temperature. After the last washing, peroxidase activity was revealed with orthophenyldiamine substrate. The reference sample used for titration curve was collected from one patient at month 6 of follow up, used at 1:200 dilution and arbitrary considered to have 1000 AU/ml (Arbitrary Units). Optical density higher than the mean +2SD of those obtained in 50 healthy controls was considered positive. Antibody titer was calculated as: (sample absorbance – blank absorbance) x 1000 / (absorbance of reference positive sample at 1/200 dilution – blank absorbance). These 3 patients showed 2323, 317, and 1830 arbitrary units/ml (AU/mL), respectively at the beginning of the follow up (FU start: day 0, 2 months, and 3 months after onset, respectively) and 955, 1162, and 2160 AU/mL respectively at the end of the follow up (FU end: 18 months, 27 months, and 29 months after disease onset, respectively).[98]

Another assay to detect anti-FHs in plasma samples was described by Goodship *et al*.[103] According to the Goodship's method (Newcastle), the blocking was performed with 200 ul of AbDserotec, and a duplicate plate was set up in absence of FH coating in which only blocking solution was added to evaluate the presence of non-FH specific readings caused by high background. Goat anti-human IgG

antibody conjugated with horse radish peroxidase (Stratech Scientific) was incubated. After washing, tetramethyl benzidine (TMB) was used as peroxidase substrate and the absorbance was detected at 450 nm. The absorbance derived from the well background in which the coating was performed with blocking solution only in the absence of FH, was subtracted from the sample absorbance obtained in presence of FH coating. The reference sample was a known positive sample provided by Dr. Dragon Durey and it was arbitrary established to have 100,000 relative units at 1/25 dilution. The antibody positivity threshold was considered to be the 97.5 percentile obtained studying 100 healthy controls and it was 350 relative units.[103]

Late, research groups of The European Working Party on Complement Genetics in Renal Disease have worked together to set up a standard procedure to evaluate anti-FHs.[104] First of all, Paris and Newcastle methods were used to study 36 plasma samples which were positive (n=12), negative (n=12), and with high background (n=12). In a second step the Jena research group repeated the assay in their laboratories using the Newcastle and Paris methods. Then all the methods of all the participants (London, Madrid, Helsinki, and Lund research groups) were tested. The last step was to evaluate the commercial assay which was performed according to the manufacturer's instructions (Abnova, Taiwan).[104]

This integrated study showed that the Paris method is highly reproducible, robust, cost effective, and easy to set up. All the methods tested gave consistent results with some exceptions caused by high background samples or when the antibody titer was very low and likely irrelevant for the disease. It was recommended to use a common positive sample for the titration curve and a common negative sample

to ensure sufficient range is present in absorbance readings. It is also important to use a sample specific subtraction to exclude the presence of false positive samples caused by hemolysed, lipidemic, or rheumatoid factors. The presence of no-serum control is also recommended to ensure the absence of contaminants. The commercial assay seems to underestimate the levels of anti-FHs and the lack of background subtraction can increase the risk of false positive samples.[104]

The consensus protocol based on the Paris method was the following:

ELISA plate (NUNC) coated with 50 ul/well of purified FH (Calbiochem) at 5 μg/mL in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) and incubated overnight at 4°C. Plate washed with PBS and then blocked with PBS/0.1% Tween/well (200 ul PBS-T) for 1 hour at room temperature. A second replicate plate should be incubated with blocking solution only. Plasma/serum samples diluted 1/50 and analyzed in triplicate. For standard curve a known positive sample diluted from 1/25 to 1/3200. Plate washed 3 times with PBS-T. Sample, negative control, positive control, and no-serum control incubated for 1 hour. Three washes with PBS-T. Incubation of 1/500 conjugated horseradish peroxidase (HRP) goat anti-human IgG for 1 hour. Three washes with PBS-T. TMB added for 5-10 min, the reaction stopped with 10% H_2SO_4 and OD450 nm readings obtained. The readings from plate incubated with only blocking solution subtracted from readings derived from the factor H coated plate.[104]

Prevalence and pathogenicity of anti-FHs

Following studies in other aHUS cohorts confirmed the presence of anti-FHs in 5-11% of aHUS cases in European cohorts, with a very high proportion of pediatric cases (prevalence in children is 13-30%).[43, 105-109] Interestingly, the prevalence of anti-FHs dramatically increased to 56.1% in aHUS cases in India.[110] Of note, anti-FHs have been described also in 1-4% of healthy subjects.[103, 111-113] Also 4 out of 21 healthy siblings reported in the Indian cohort by Sinha *et al.* showed high antibody titers ranging from 497.9 to 1356.6 AU/mL.[110]

The anti-FHs were detectable in plasma/serum samples collected in both acute or remission phases of the disease. Especially during the acute phase anti-FHs can circulate with FH as immune complexes which can also be detected by ELISA assay.[114]

Plasma samples from patients with anti-FHs were able to induce the lysis of sheep erythrocytes which was proportional to the antibody titer. The hemolysis was reduced with the addition of FH or using IgG depleted serum. *In vitro* studies demonstrated that the purified anti-FH IgGs can lead to a reduced FH binding to C3(H_2O), C3c, and C3d and can also decrease the ability of FH to promote the C3 convertase decay. The Fab'2 fractions also inhibited the FH activity. Anti-FHs can modify the FH cofactor activity for the cleavage of C3b by FI.[109, 114, 115]

Clinical features and complement profile of patients with anti-FHs

Anti-FHs affected mainly children and the age at the disease onset was reported to be between 5 and 15 years.[109] It is different from the disease onset described in aHUS patients carrying mutations in *CFH* or *DGKE* mutations that occurs mainly

in the first years of life.[62, 100] On the contrary, it is more similar to the disease onset described in patients with *CD46* mutations.[62, 109] Only few adult cases with anti-FHs were reported in literature.[62, 106] No gender difference was described.[106]

Most aHUS cases with anti-FHs presented prodromal signs. The large majority showed fever, upper respiratory tract infections or gastrointestinal signs (such as vomiting, diarrhea, and abdominal pain). Prodromal varicella, norovirus, STEC and *Plasmodium vivax* infections have been also reported.[106, 110] Other patients showed urticaria, transient face edema, and seizures at the disease onset.[106]At the time of hospitalization, most patients presented thrombocytopenic anemia, oligoanuria, hematuria, and severe hypertension. As reported by Dragon-Durey *et al*., hepatitis and pancreatitis were observed in many patients and anti-nuclear antibodies were found in 24% of tested patients, but this association was not confirmed in other studies.[106]

Low C3 levels were reported in about half of patients with anti-FHs indicating the AP of complement was activated. C4 was normal in the large majority of them. The FH antigen levels were not reported to be correlated with the anti-FH titer despite in the acute phase of the disease decreased in few patients.[109]

Outcome, therapy and transplant

Atypical HUS relapses have been reported in a variable number of patients with anti-FHs in different studies (ranging from 13% to about 50%) and in the large majority of cases occurred within one year from disease onset.[109] Dragon-Durey *et al.* reported that 27% of patients developed ESRD during a follow up of 39 months.[106] Sinha *et al.* also observed a high prevalence of renal failure in 33.6%

of patients at the last follow up.[110] Plasma infusion was adopted in some patients but plasma exchange was more effective to induce hematological remission because it can remove autoantibodies from the circulation and provide free FH (but also FHR1) that can compete with FH for the antibody binding.[116] Despite plasma exchange treatment, about 35% of aHUS patients with anti-FHs developed ESRD or died.[109] Moreover, after plasma exchange, the antibody titer can increase with a high risk of relapses. For this reason prolonged immunosuppression is often used (prednisone, cyclophosphamide, rituximab, and mycophenolate mofetil, with different protocols) to prevent or control the new formation of anti-FHs. The combined plasma exchange and immunosuppression treatment has been reported to be more effective therapy to protect renal function and to prevent aHUS relapses than plasma therapy alone (only 14.3% of patients with combined therapy developed ESRD or died).[109, 110, 117]

A few aHUS cases with anti-FHs, in which Eculizumab therapy was adopted, resulted in the remission of the disease, discontinuation of plasma exchange, and renal recovery in the majority of patients.[118-120] However, Eculizumab cannot prevent the generation of anti-FHs and thus combined immunosuppressive treatments are needed.[109]

TMA recurrence after kidney transplantation was described in about 20% of aHUS cases with anti-FHs (total transplanted patients, n=21).[109] In the group of aHUS patients with anti-FHs who received a preventive therapy based on plasma exchange and immunosuppression before the transplantation (n=9), only one patient developed TMA recurrence on the graft and two patients lost the graft for

acute rejection.[109] Eculizumab was used as successful approach to prevent the disease recurrence on the graft in two cases.[109]

Antibody isotypes and FH epitopes recognized by anti-FHs

Antibody isotypes in patients with aHUS included mainly IgG and less frequently IgA. The identified IgG subtypes were IgG3 (present in 60-100% of tested samples) and IgG1 (rarely alone but present in 30% of tested samples in combination with IgG3).[109]

Anti-FHs bind multiple FH epitopes mainly located at the FH C-terminal domain which is a mutation hotspot in aHUS, indicating that both anti-FHs and *CFH* genetic abnormalities have similar functional consequences on FH regulatory activity on cell surfaces.[115] Anti-FHs recognized FH SCRs 15-20 in 97% of tested samples (63/65) and FH SCRs 19-20 in 77% of tested samples (48/62), as reported in literature.[109] On the contrary, among 21 tested samples, none showed a binding activity to FH SCRs 15-19, indicating that the main target domain of anti-FHs is the FH SCR20.[109] Bhattacharjee *et al.* identified the autoantibody epitopes on FH SCR20 using FH mutants at the residues Thr1184, Lys1186, Lys1188, Leu1189, and Glu1198 which overlap with the heparin and glycosaminoglycans cell binding sites and also the amino acids used by microbes to bind FH and evade the immune system.[121] Trojnar *et al.* performed a linear epitope mapping using synthetic FH peptides and validating the results with recombinant FH carrying point mutations. The results showed the anti-FHs specifically recognized FH epitopes at 1157-1171 (SCR 19), 1177-1191 and 1207-1226 (both in SCR 20) residues. The linear FH binding sites are located close to each other in the protein

tertiary structure, suggesting that the antibody binding site can be the result of specific FH conformation during the protein folding.[122]

Although FH C-terminus is the main domain recognized by autoantibodies, others FH domains can be recognized. FH N-terminal SCRs 1-4 have been found to bind antibodies in 36% of tested aHUS samples (15/42) while SCRs 1-7 and SCRs 8-14 are recognized in 36% (23/63) and 21% (12/56) of samples.[109]

Genetics of anti-FH mediated aHUS

The most important genetic finding in the study of the anti-FH mediated aHUS is the association with the FHR1 deficiency that is present in about 80-90% of aHUS patients with anti FHs.[43, 105, 107, 108, 110, 123] For this reason the autoimmune form of aHUS was also defined as DEAP-aHUS, which means *De*ficiency of FHR plasma proteins and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome.[124]

The large majority of aHUS patients with FHR1 deficiency was found to carry the homozygous *CFHR3-CFHR1* deletion, which is a common polymorphism that has variable frequencies among human populations.[42] A high frequency of the deleted allele is observed in DNA samples of healthy controls from South-Saharan Africa and Nigeria (33.7% and 54.7%, respectively), it is around 15-25% in European Caucasoid populations and very low in East Asia and South America.[42] This means that about 30% of Nigerian, 3-10% of the European, and about 0% in East Asia and South America are homozygotes for the deleted allele. About 15% of aHUS patients with anti-FHs were compound heterozygous for the *CFHR3-CFHR1* and *CFHR1-CFHR4* deletion.[43, 107] The *CFHR1-CFHR4* deletion was detected in about 0.9% of healthy subjects and it was not reported to be

abundant in aHUS patients with anti-FHs, but the concomitant presence of *CFHR3-CFHR1* and *CFHR1-CFHR4* deletions is significantly more frequent in aHUS patients with anti-FHs compared to healthy individuals. This observation suggests that the complete *CFHR1* lack is the crucial genetic factor associated with the production of factor H autoantibodies in aHUS. Further confirmation is given by the fact that other very rare patients have shown the *CFHR3-CFHR1* deletion combined with nonsense *CFHR1* mutation, which leads to FHR1 truncated protein, or the homozygous *CFHR1-CFHR4* deletion.[43]

Whether the few healthy controls with anti-FHs reported in literature carry the homozygous *CFHR1* deletion is not known, but all the four healthy siblings with high anti-FH titers described in Sinha *et al.* presented the homozygous *CFHR1* deletion.[110] This finding could indicate that the *CFHR1* deletion could have a main role in the predisposition to anti-FHs, although the presence of anti-FHs in healthy subjects could suggest that additional factors are required for the disease to manifest.

The deleted *CFHR3-CFHR1* allele was also reported to be a risk factor for SLE and hematopoietic stem cell transplantation-related thrombotic microangiopathy.[112, 125] On the contrary it was a protective allele for AMD and IgA nephropathy.[126, 127] Interestingly, anti-FHs mediated aHUS was more abundant in India than in Europe but this regional difference did not reflect the *CFHR1* deletion frequency, which was rarer in India than in Europe. Furthermore, in Nigeria where the homozygous *CFHR3-CFHR1* deletion is present in 30% of the population an increased risk of aHUS has been not been described. At variance, in IgA nephropathy, a direct correlation between the prevalence of the

disease and the frequency of the *CFHR3-CFHR1* deletion was observed. Indeed, IgA nephropathy was more common in East Asia where the protective allele is very rare and less common in Africans in whom the protective allele is abundant.[34]

The pathogenic mechanism that links the FHR1 deficiency with the risk of anti-FH development is still under investigation. Atypical HUS associated anti-FHs cross-reacted with FHR1 SCRs 4-5 which show a very high amino acid sequence identity with SCRs 19-20 of FH.[116] In this region indeed the only two amino acid differences are Leu290 (that corresponds to FH Ser1191) and Ala296 (that corresponds to FH Val1197). The addition of FHR1 reduced the sheep erythrocyte hemolysis in a dose-dependent manner during the hemolytic assay in presence of purified anti-FHs from aHUS plasma, an effect similar to that of the addition of FH.[116] FH and FHR1 were both recognized by autoantibodies but the backbone and the loops of the auto-antigenic epitopes localized in FH SCRs 19-20 (Arg1182-Leu1189) and FHR1 SCRs 4-5 (Arg281-Leu288) showed a different orientation at X-ray. The main difference is the formation of a prominent alpha helix in the loop Arg281 through Leu288 of FHR1 SCR5 while in the corresponding FH SCR20 region it was not observed. Interestingly, an altered conformation of the loop Arg1182 through Leu1189 of FH SCR20 is observed when FH was bound to its natural ligands (such as *Borrelia* OspE protein, sialic acid glycan, and C3b). This finding has suggested a model to explain the origin of the autoantibodies toward FH. It is possible that some of the microbial proteins, that bind FH SCRs 19-20, can induce a conformational modification of FH molecule which is similar to that spontaneously reached by FHR1. Whether the

FH epitope bound by the microbial protein is not masked during this binding it can generate the formation of an autoantigenic neoepitope which can lead to the risk of autoimmunity in the absence of FHR1. Indeed, in individuals with FHR1, an FHR1 epitope similar to the hypothetical FH autoantigenic epitope is present and it can guarantee the tolerance to FH.[121]

In addition to FHR1 deficiency, other defects in genes encoding complement proteins have been reported in about 15% of aHUS patients with anti-FHs[43, 105, 107, 109, 128, 129].

Heterozygous mutations in *CFH* were found in 5 patients (5% among 95 tested patients). The *CFH* c.2850G>T, p.Gln950His was reported in 2 of these patients (other genetic variants were not specified). Heterozygous *CD46* mutations have been described in 2 patients with anti-FHs (2%, among 95 tested patients) and one of them did not show the FHR1 deficiency (carrying the *CD46* c.718T>C, p.Ser240Pro). Heterozygous *CFI* mutations were described in 3 patients (3%, among 95 tested patients) and 2 of them showed the absence of FHR1 (in these last 2 patients the *CFI* mutations were c.1071T>G, p.Ile357Met and p.1216C>T, p.Arg406Cys). Three patients were carriers of *C3* heterozygous mutations namely c.-3_-2dup and c.1898A>G, p.Lys633Arg in combination with the homozygous *CFHR1* deletion in two patients, and c.481C>T, p.Arg161Trp alone in the third patient. Heterozygous *CFB* mutations were not reported in literature in combination with anti-FHs. Of note, a patient with anti-FHs showed a heterozygous mutation in *THBD* and another one was a compound heterozygotes for FXII gene mutations c.9277T>G, p.Trp222Gly and c.10468C>A, p.Arg447Ser which lead to a complete FXII deficiency. Most of the above mutations have

functional effects by *in vitro* assays or are predicted as damaging by *in silico* analyses. [109]

Not enough studies have been done to clarify the role of common variants in complement genes in the predisposition to anti-FHs mediated aHUS. Moore *et al.* reported the presence of $CD46_{GGAAC}$ haplotype in 10 out of 13 patients with anti-FHs while only one presents the *CFH* H3 haplotype.[107] Interestingly, the authors also reported that 12 out of 13 aHUS patients with anti-FHs carried the *CFH* "A" variant at the rs3753396 and "G" variant at rs1065489 suggesting a possible role of particular *CFH* haplotypes in predisposing to development of anti-FHs.[107]

1.10 Anti-FHs in other diseases

Anti-FHs antibodies have been reported in 23 patients affected by C3G or MPGN.[103, 109, 130] Most C3G/MPGN patients were adults and showed low C3 levels. In 30% of them anti-FHs were present in combination with C3NeF, which are auto-antibodies directed against AP C3 convertase C3bBb, preventing the Bb dissociation and leading to a more stable C3 convertase.[109] Anti-FHs in C3G/MPGN patients recognized the FH N-terminal domains (SCRs 1-5) although additional binding sites have been found in the central and C-terminal domains. These findings are at variance with anti-FHs associated with aHUS which mainly target the C-terminus surface recognition domain of FH. Anti-FHs purified from C3G/MPGN patient samples were not able to decrease the binding of FH to C3, C3c, and C3d as occurred with anti-FHs derived from aHUS patients. Blanc *et al.* detected very low levels of FH-anti-FH immune complexes in C3G/MPGN patients.[130] Taken together these data suggested that anti-FHs from C3G/MPGN

patients present a weaker avidity for FH compared to anti-FHs from aHUS patients. Interestingly, the homozygous *CFHR1* deletion, which is found strongly associated with anti-FHs in aHUS, was not reported to predispose to anti-FH development in C3G/MPGN.[103, 130]

Anti-FHs were also reported in a 17 year-old Caucasian woman with very low levels of ADAMTS13 and FH caused by anti-ADAMTS13 and anti-FH antibodies, in whom a combined diagnosis of thrombotic thrombocytopenic purpura (TTP) and HUS was done.[131] Anti-FHs were also found in a consistent percentage of patients with rheumatic diseases, such as RA and SLE, in few patients with non-small-cell lung cancer, and in individuals who have developed TMA post hematopoietic stem cell transplantation.[111, 112, 132] Recently, Senant *et al.* have shown anti-FHs in inflammatory myopathies (50% of patients with pediatric myositis and 43% of adults with myositis).[133] Of note, the homozygous *CFHR1* deletion was not reported in association with anti-FH formation in all above diseases with the only exception of aHUS.

1.11 Immunologic tolerance

Lymphocytes are the principal components of the adaptive immunity. They are specialized for the recognition and the response to foreign antigens. Specifically, B lymphocytes are the only cells able to produce antibodies which recognize soluble antigens while T lymphocytes have membrane receptors (TCRs) which can interact with peptides derived from intracellular microbes displayed on host MHC (Major Histocompatibility Complex) molecules present on APCs. T lymphocytes are classified by different cell populations: 1) CD4+ Helper T cells which are specialized in secreting cytokines that stimulate the proliferation and differentiation of T cells themselves, B cells, and macrophages; 2) CD8+ cytotoxic T lymphocytes which kill cells infected by viruses; 3) regulatory T lymphocytes (Tregs), which can inhibit the immune response.

In the thymus and bone marrow, the DNA encoding TCR and B cell receptor (BCR) of the immature T and B lymphocytes, respectively, randomly recombine and some rearrangements can obviously generate receptors able to recognise self-antigens determining a potential risk for autoimmunity. To avoid this problem, immunologic tolerance has evolved in the adaptive immune system. Immunologic tolerance is defined as the unresponsiveness of B and T lymphocytes to an antigen induced by its previous exposure and is mediated by central and peripheral control mechanisms. Central tolerance leads to the maturation of lymphocytes which do not respond to self-antigens and eliminates immature lymphocytes which react against self-antigens. Since some self-reactive cells can escape the central tolerance control, a peripheral tolerance is needed to induce the apoptosis of escaped self-reactive lymphocytes, to prevent the activation of these potentially

reactive lymphocytes, and also to induce the tolerance to self-antigens that are expressed only in adults. Tregs cells are also important suppressors of auto-reactive T cells contributing to the peripheral tolerance.[134]

T lymphocyte central tolerance

During the maturation in thymus, T lymphocytes with TCRs recognizing with high avidity self-antigens, which are presented on class I or II MHC molecules expressed on dendritic or epithelial cells in thymus, are induced to apoptosis and deleted (negative selection). Moreover, some of the T lymphocytes that recognize self-antigens are committed to the CD4+ lineage developing into Treg cells.

In the thymus all the self-antigens of our organisms are represented: some of them are ubiquitously expressed, others are taken in from the blood circulation and others, which are tissue specific antigens (TSAs), such as insulin, are expressed in thymus by specialized medullary thymic epithelial cells (mTECs) through an elegant mechanism mediated by the *A*uto*I*mmune *RE*gulator (AIRE).[134]

The human AIRE is a transcriptional factor which acts as a coactivator in a large transcriptional complex promoting the expression of more than 3000 different proteins, especially TSAs in thymus mTCEs.[135] AIRE is a protein of about 56 kDa characterized by five domains: an oligomerization domain called CARD, the Nuclear Location Signal crucial for the migration of the protein to the nucleus; the SAND domain involved in the protein-protein interaction and two PHD domains, which are important in the interaction with chromatin and in its decondensation.[136] Mutations in *AIRE* have been found in patients affected by "Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy", APECED, also called

"Autoimmune Polyendromic Syndrome type 1" (APS1). The disease is characterized by development of auto-antibodies against multiple targets and lymphocyte mediated infiltration in several endocrine organs.[136] In *Aire*$^{-/-}$ mouse model, the TSAs expression in thymus decreased, auto-antibodies against self-antigens were detected, Treg population decreased, and activated self-reactive T cells were present in periphery leading to a general autoimmune reaction similar to that found in APS1 patients.[136] The expression of AIRE is finely regulated by both negative and positive mechanisms and this leads to its expression in only a small percentage of mTECs (1–3%).[135] Through its PHD domain, AIRE seems to recognize epigenetic marks at the promoter of TSAs, such as H3K4me and H3K27me3, and acts forming a complex with other transcriptional activators (such as pTEFb complex, CBP, DNA-PK, TOP2A, and SIRT1) which leads to chromatin modifications, to the recruitment of RNA polymerase II, and to the beginning of the transcription. Kumar *et al.* described that AIRE dimers and tetramers can recognize DNA sequence containing G-doublets with the ATTGGTTA motif and the TTATTA box. Through competition assays the authors demonstrated that sequence with one TTATTA motif and two tandem repeats of ATTGGTTA had the highest binding affinity for AIRE.[137]

The expression of TSAs in the thymus and its relevance in autoimmune diseases have been demonstrated in genetic studies in which gene variants that affected thymus expression of human insulin and acetylcholine receptor determined susceptibility T1D and myasthemia gravis, respectively.[138, 139]

T lymphocyte peripheral tolerance

In addition to the central tolerance, mechanisms such as anergy, suppression mediated by Treg cells, and apoptosis can maintain the immunologic tolerance also in periphery.[134]

- *Self-reactive T cells become anergic*

The full activation of peripheral T cells requires the recognition of self-antigens by TCR and also the production of costimulatory signals by APCs (such as B7-1/CD80 and B7-2/CD86) which bind CD28 receptor on T cells. If the TCR binds self-antigen-MHC complex for prolonged time in the absence of CD28 activation, T cells become unresponsive to that antigen leading to anergy.[134]

The anergy can be obtained not only with the absence of the costimulatory molecules but also with the engagement of inhibitory receptors on T cells, which also have an important role in self-tolerance. CTLA-4 is an inhibitory receptor which belongs to the CD28 receptor family and binds B7 molecules with higher affinity compared to CD28. It is expressed on CD4+ T cells and it is important to control the initial activation mediated by antigen-MHC-TCR interaction in lymphoid organs. It is also expressed on Treg cells and mediates their suppressive activity.[140] Knockout mice for *CTLA-4* develop uncontrolled T cells activation and an autoimmune phenotype.[141] Complete loss of CTLA-4 leads to fatal autoimmune disease in mouse models and mutations in *CTLA-4* are associated in humans with several autoimmune diseases, such as T1D, and Grave's disease.[142, 143] PD-1 is another inhibitory receptor of CD28 family encoded by *PDCD1* gene and expressed on T cells and on B cell progenitors, which recognizes two different ligands (PD-L1 and PD-L2) present on APCs. PD-1 interaction with PD-

L1 or PD-L2 determines the inactivation of T cell responses to antigen stimulation through the inhibition of T cell proliferation and cytokine production. PD-1 is crucial to inactivate the peripheral immune responses of effector T cells. PD-1 deficient mice developed autoimmune diseases, such as SLE and arthritis. In humans, polymorphisms in *PDCD1* are associated to several autoimmune diseases, including SLE, T1D, MS, RA, Grave's disease and ankylosing spondylitis (AS).[134, 143-146]

- *Self-reactive T cells are suppressed by Treg cells*

Treg cells are CD4+, Foxp3+, $CD25^{high}$ (interleukin-2 receptor α chain, IL-2R) T cells and their main role is to maintain the self-tolerance suppressing the proliferation of naive T cells and their differentiation to effector T cells. They can also suppress effector activities of differentiated CD4+, CD8+ T cells and natural killer cells.[147]

Foxp3 is a transcription factor that is crucial for Treg cell development and functions, and a murine model with mutations in *Foxp3* showed multisystemic autoimmune disease associated with the absence of Treg cells.[147] In humans *Foxp3* mutations are associated with a rare autoimmune disorder, called IPEX syndrome, that means "Immune dysregulation, polyendocrinopathy, enteropathy, X linked" and with a deficiency of Treg cells.[148]

Knockout mice lacking IL-2 or IL-2R chains developed autoimmune hemolytic anemia, multiple autoantibodies, and inflammatory bowel disease.[147] Moreover, in these animals the Treg population is not present and the disease phenotype can be corrected restoring Treg cells.[147] These data indicated that IL-2 is essential for the

Tregs differentiation and maintenance. Treg cells express high levels of CTLA-4 that is crucial for their suppressive function which are mediated by the production of immunosuppressive cytokines, such as IL-10 (that inhibits the expression of costimulatory molecule, class II MHC, and the production of IL-2 by APCs), and TGF-β (that inhibits the proliferation and effector functions of T cells, regulates the differentiation of CD4+ toward inflammatory T_H17 cells, and also guarantees the Treg cell maintenance). Elimination of TGF-β signals determines a systemic inflammatory response because of uncontrolled T cell activation and lack of Treg cells.[147] A mouse model where IL-10 was knocked out developed inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis.[149] Moreover, Tregs can directly inhibit the activation of CD8+ T cells or suppress CD4+ helpers that are required for the full activation of CD8+ T cells.[134]

- *Self-reactive T cells die by apoptosis*

When TCR recognize self-antigens with high avidity in the absence of costimulatory molecules, T cells can activate a specific sensor protein called Bim which interacts with pro-apoptotic proteins belonging to the Bcl-2 family (Bax and Bak) and promotes the activation of cell apoptosis by the mitochondrial pathway. In the same way, when T cells are repeatedly stimulated, FasL is secreted and interacts with its receptor Fas (CD95) which activates the caspase cascade leading to apoptotic cell death. Murine models carrying mutations in Fas or FasL developed systemic autoimmune disease similar to SLE. In humans, defects in Fas-FasL pathway cause the autoimmune lymphoproliferative syndrome (ALPS), which is characterized by lymphoproliferation, and secondary cancers.[134, 150]

B lymphocyte tolerance

B cell tolerance is important to prevent immunologic response against self-antigens, also including self-polysaccharides and lipids, for which the tolerance is not mediated by thymus. An important mechanism to maintain the central B cell tolerance is the receptor editing. When BCRs recognize with high avidity self-antigens present in bone marrow, immature B cells can upregulate RAG1 and RAG2 genes which are devoted to the VJ sequence recombination in Ig k light chain genes leading to the expression of a new BCR on cell surface which does not recognize the self-antigen. If the new rearranged VJ sequences are not productive, the immature B cells activate the apoptotic pathway. On the contrary, when BCRs recognize with weak avidity the self-antigens, B cells become anergic. In periphery, B cells, which bind self-antigens with high avidity and are repeatedly stimulated in absence of specific T helper activation, become anergic or die by apoptosis. If a peripheral B cell recognize a self-antigen with low avidity, inhibitory receptors, such as CD22 are engaged to attenuate BCR signaling, preventing B cell activation.[134]

1.12 Autoimmunity

Loss of tolerance to self-antigens causes autoimmune diseases. Autoimmune diseases affect many young individuals and represent a significant clinical problem because of their chronic and progressive nature and associated healthcare costs.[151] Most of the current therapies can resolve the concomitant inflammation and can block the activation of self-reactive immune cells, but they cannot correct or prevent the loss of tolerance.[151]

In autoimmune diseases, hyper reactive T and B lymphocytes are often observed in combination with autoantibodies. In monozygotic twins the chances to develop an autoimmune disease is higher than in dizygotic twins suggesting that genetic factors are involved in predispose to the autoimmunity. Moreover, in the families with multiple affected subjects, the probability for the healthy family members to develop different autoimmune diseases is increased, suggesting the presence of genetic defects in general mechanisms of immunological tolerance which can predispose to several kind of autoimmune diseases. Rare autoimmune diseases are usually monogenic and are caused by mutations with a strong effect which impair tolerance pathways. IPEX and APECED are two of such examples determined by mutations in *FOXP3* and *AIRE* genes, as described in the previous section of this introduction. Instead, common autoimmune diseases are complex disorders in which multiple genetic and environmental susceptibility variants are involved.[152, 153]

Genome wide association studies (GWAS) have identified hundreds of loci involved in common autoimmune diseases.[151] The contribution of each variant is usually small and it is likely that the presence of multiple variants can favor the development of the disease.[151] The single risk variant can be often found also in healthy subjects although at lower frequency than in patients and without the combination with other predisposing variants. Furthermore, some of these genetic variants are associated with different autoimmune diseases, suggesting that alteration of common pathways may play a role.[151] Risk variants for autoimmune diseases are often located in non-coding regions suggesting their involvement in

the regulation of gene transcription, protein expression, or epigenetic modifications.[153]

Genetic mutations or polymorphisms can lead to defects during the negative selection in T and B cells, during the BCR editing, in Treg cell functions, in apoptotic pathway of self-reactive lymphocytes, and in inhibitory receptors. These defects can determine a reduction of the lymphocyte activation threshold leading to a susceptibility state for the loss of self-tolerance.[152]

The strongest association with autoimmunity has been found with human leukocyte antigen (HLA) loci which contribute to half or more of the genetic susceptibility in most autoimmune disorders.[154]

Besides HLA, one of the most common genetic associations to autoimmune diseases is the rs2476601 (c.1858C>T, p.Arg620Trp), a non-synonymous SNP in *PTPN22* which encodes a tyrosine phosphatase expressed in lymphocytes. The 620Trp variant determines a significant alteration in TCR and BCR signaling and is reported to be strongly associated with T1D, autoimmune thyroiditis, SLE, Grave's disease, vitiligo, and RA.[153, 155]

Other loci associated with autoimmunity include genes encoding molecules expressed by lymphocytes and involved in costimulatory functions. For example, CD6 is a transmembrane glycoprotein of T and B lymphocytes. It binds the activated leukocyte cell adhesion molecule (ALCAM) expressed by APCs, leading to activation of the costimulatory cellular pathway. De Jager *et al.* have described an increased risk to develop MS in individuals carrying the CD6

rs17824933 G variant which determines the production of a shorter CD6 isoform that cannot bind ALCAM. [153, 155, 156]

Others susceptibility variants have been found in cytokines and cytokine receptor genes. Polymorphisms in IL-23R represent susceptibility factors for inflammatory bowel diseases and psoriasis. Indeed, IL-23 is an important cytokine that induces T_H17 cell differentiation toward a pro-inflammatory phenotype.[134] Tumor necrosis factor receptor superfamily member 1A, TNFRSF1A, can activate the transcription of pro-inflammatory factor NF-kB and the cellular apoptotic pathway. A polymorphism in the splicing acceptor site in exon 6 of *TNFRSF1A* has been reported in strong association with MS because it determines the formation of a shorter protein that enhances NF-kB activation and higher response to inflammatory stimuli.[155, 156] Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a protein expressed in the peripheral blood leukocytes but also in intestinal epithelial cells able to recognize bacterial molecules and to activate NF-kB pro-inflammatory pathway. Defects in *NOD2* can leads to an inefficient immune response against intestinal microbes causing inflammatory bowel diseases, such as Crohn's disease.[134]

Infections can trigger autoimmune diseases activating the innate immune system which is *per se* a strong stimulus for adaptive immunity. Moreover, infections and tissue damages can promote the recruitment of self-reactive lymphocytes to the site of inflammation increasing the risk of their activation. After an infection, some self-antigens can be displayed to immune cells in a different fashion showing neo epitopes which can be recognized as non-self- antigens. Enzymatic modifications, increased expression and failure of clearance can render the self-

antigens more immunogenic and can also protract the duration of the activating stimulus causing an increased risk of autoimmunity.[134] Of note, infections can also induce autoimmunity via molecular mimicry of microbial proteins with self-antigens.[157] Interestingly, some infections paradoxically can protect from autoimmunity, as demonstrated in T1D and MS.[134] Finally, the intestinal and cutaneous microbioma may also influence the development of autoimmune diseases.[134]

1.13 The HLA system

The major histocompatibility complex (MHC) locus, also known as the leukocyte antigen (HLA) locus, spans around 4 Mbp on the short arm of chromosome 6 and contains both class I and class II MHC genes and also other genes involved in antigen presentation (Figure 1-5).[154]

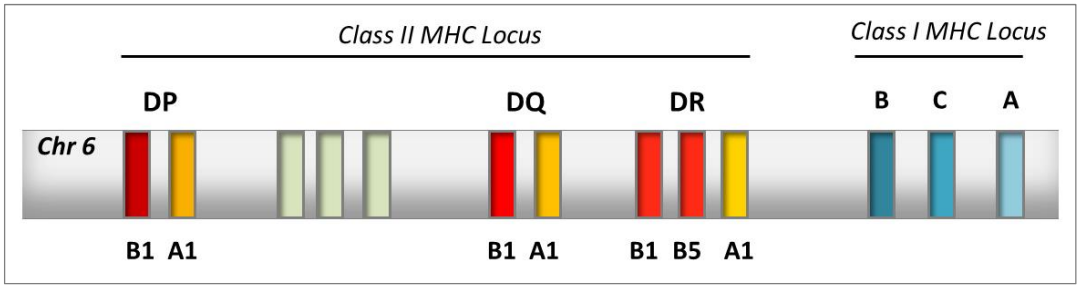

Figure 1-5. Schematic map of MHC loci on human chromosome 6.

MHC class I genes (A, B, and C) and MHC class II genes (DP, DQ, and DR) are shown. The A1 genes, encoding the alpha chains of class II MHC molecules, are represented in red and the B1 genes, encoding the beta chains of class II MHC molecules, are represented in yellow. Here, a specific DR haplotype is represented with the *HLA-DRA1*, the *HLA-DRB1,* and the *HLA-DRB5* genes.

Class I MHC genes include HLA-A, HLA-B, and HLA-C (Figure 1-5), each encoding an α chain which is non-covalently linked to a non-MHC subunit, called β2-microglobulin, forming the class I MHC molecule (Figure 1-6).

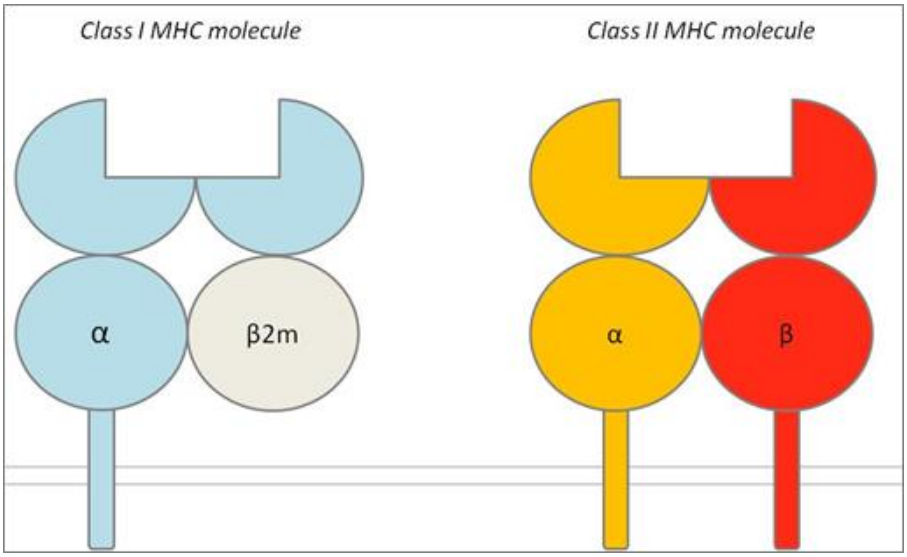

Figure 1-6. Schematic structures of class I and class II MHC molecules.

Class I MHC molecule is constituted by α chain and β2-microglobulin. Class II MHC molecule is formed by the α and the β chains.

Class II MHC genes include HLA-DR, HLA-DQ, and HLA-DP (Figure 1-5). Each class II MHC molecule forms a heterodimer composed by an α chain and a β chain derived from A1 and B1 genes, respectively (for example an α chain from HLA-DQA1 and a β chain derived from HLA-DQB1, Figure 1-6). The β chain of a HLA-DR molecules is encoded by the gene product of *HLA-DRB1* or the paralogue (*DRB3*, *DRB4*, or *DRB5)*. Each individual presents two haplotypes and each haplotype generally contains one *HLA-DRA1*, one *HLA-DRB1* plus one additional *HLA-DRB(3-5)* paralogue (Figure 1-5).

The MHC genes are the most polymorphic genes in the whole human genome caused by the presence of several alleles (more than 5,000 different alleles described) and not caused by gene recombination (such as in T or B cell receptors). MHC molecules are also codominantly expressed in each individual. The polygenic structure, the polymorphisms, and the codominance determine the

presence of a high number of different MHC molecules on APC cell surfaces and this represents a sophisticated approach to bind several different antigens and display them to CD8+ or CD4+ T cells. The complex and refined MHC system structure allows, first of all, to educate the immune system to tolerate all the self-antigens present in the organism, and also to guarantee the immune defense against the greatest number of pathogens.[134]

Each MHC molecule is a transmembrane protein formed by an extracellular peptide cleft, an immunoglobulin (Ig)-like domain, a transmembrane domain, and a cytoplasmic domain. The cleft is composed by the N terminal amino acids of MHC chains which are located in 2 α helices forming the two walls and an 8 stranded β-pleated sheet that form the floor of the groove. The polymorphic variants are located both in the walls and in the floor of the cleft.[134]

Class I MHC

Class I molecules are formed by two non-covalently associated polypeptide chains, the α chain (about 44-47 kDa) and β2 microglobulin (12 kDa). The α chain N-terminus is constituted by the extracellular domain while its C terminus is cytoplasmic. At N-terminal domain, two segments called α1 and α2, form the peptide binding cleft which carries the polymorphic variants. Another extracellular segment, called α3, contains the Ig domain which is conserved between all the class I MHC molecules and together with β2 microglobulin, is important for the interaction with the CD8 co-receptor on T cells. The MHC class I groove can bind only small peptides between 8 and 11 amino acid long in a flexible extended conformation because of its closed ends at the peptide binding

cleft. The assembled class I molecule is a trimer formed by α chain, β2 microglobulin, and the bound peptide. The stable expression of this complex requires the presence of all these three components. Class I molecules are expressed on all nucleated cells which can be potentially infected by viruses or become tumors. CD8+ cytotoxic lymphocytes can recognize class I MHC expressing cells and kill them when are infected by intracellular microbes, like viruses, or when they are transforming themselves in tumors. Most individuals are heterozygous and express both alleles for each class I MHC gene. For this reason six different class I MHC molecules are present on cell surfaces of each individual.[134]

Class II MHC

Class II molecules are formed by two non-covalently linked polypeptide chains, α chain (32-34 kDa) and β chain (29-32 kDa). The N-terminal domains of both chains (called α1 and β1 segments) form the peptide binding cleft that is structurally identical to the class I MHC groove and contains the polymorphic variants, mostly in the β chain. The molecular structure of the class II MHC groove allows the accommodation of larger peptides than class I MHC with a weight around 30 amino acids or more. The α2 and β2 segments of class II molecules are folded into Ig domains and are non-polymorphic. Class II MHC molecules are expressed on APCs, such as dendritic cells, B lymphocytes, and macrophages. Extracellular antigens are endocytosed into vesicles in APCs, processed and displayed by class II MHC molecules which are recognized by CD4+ T cells.[134]

Genetics of HLA in autoimmune diseases

Using SSP (Sequencing-Based Typing), SSO (Sequence Specific Oligonucleotide probes) and NGS (Next Generation Sequencing) technologies, in which locus/exon specific primer mixes are used, the accuracy of the MHC molecular typing has been improved compared to serologic tests or molecular typing based on the amplification of specific allele or allele families (SSP, Sequence Specific Primer method), leading to improved definition of which HLA alleles are associated with several autoimmune diseases. Several studies have demonstrated that some HLA alleles are present with a higher frequency in patients affected by autoimmune diseases than in the healthy individuals, increasing the odds ratio (OR) to develop the diseases in subjects who are carriers of these risk alleles. Some examples are HLA-B*27:02 and B*27:05 in AS, HLA-DRB1*01:03 in celiac disease, and HLA-DRB1*15:01 in MS and SLE indicating that both class I and II MHC alleles are implicated in autoimmune predisposition. In addition, some autoimmune diseases shared the same HLA risk allele suggesting the presence of common altered pathways. In some diseases, such as T1D, both class I and class II risk alleles have been associated, indicating that both CD8+ cytotoxic and CD4+ helper T-cells are involved in the pathogenesis.

Several alleles in MHC locus are inherited together because of the presence of a strong linkage disequilibrium (LD) existing in this genomic region and this can lead to a more difficult interpretation of the results.[158] Sometimes a disease can be found in association with a specific HLA allele, but the causative allele could be another one that is co-inherited in the same HLA haplotype.[154] Furthermore, epistasis has been reported in many autoimmune diseases, such as SLE, MS, and

AS in which HLA and non-HLA genetic defects were observed, suggesting that more defects in the same pathway have to be present for the disease to manifest as it has been commonly described in complex diseases.[154] Interestingly, others HLA alleles have been associated with protection from autoimmune disease also in the presence of predisposing alleles.[154]

GWAS has allowed identification of the key amino acids that are shared by different MHC molecules associated with a higher risk of developing autoimmune diseases.[154] A good example is RA in which three amino acids found in several HLA-DRβ1 molecules in combination to two amino acids, one in HLA-B and one in HLA-DP, conferred an increased risk for the disease. The amino acids are located within the peptide-binding cleft indicating their crucial involvement in antigen recognition during T cell maturation in the thymus or peripheral activation.[159]

The mechanisms underlying MHC involvement in the autoimmunity remain poorly understood. MHC class II alleles have been found to be most strongly associated with autoimmune diseases compared to class I MHC alleles suggesting that the loss of self-tolerance may be caused by defective antigen presentation by class II MHC molecules on epithelial or dendritic cells to self-reactive T cells.[154] The peptide binding proprieties depend from the peptide size and the non-covalent interactions with polymorphic amino acids present in the binding cleft of MHC molecules which are determined by the presence of specific HLA alleles.[134] During the negative selection in thymus, T cells whose TCRs strongly recognize self-peptide-MHC complex are deleted. A weak affinity between self-peptides and MHC molecules can determine structural alterations in TCR-peptide-MHC

complex that compromise its overall stability leading to the loss of immunological tolerance of self-antigens.[160, 161]

2 Aims

The global aim of the present thesis was to evaluate the contribution of genetics to the development of anti-FH antibodies in patients affected by aHUS.

The first part of this thesis was focused on the development of an accurate ELISA assay to detect anti-FHs in patient samples and to evaluate the prevalence of anti-FHs in our aHUS cohort. Another aim was to investigate the prevalence of the FHR1 deficiency in a large group of aHUS patients and controls and to evaluate the association between the presence of the FHR1 deficiency and the risk to develop anti-FH associated aHUS. Moreover, the evaluation whether additional genetic factors (mutations or polymorphisms) in complement genes can increase the risk to develop anti-FH associated aHUS was another important objective. Of note, the association with common variants was investigated using as reference group "super controls", *i.e.* healthy subjects with the homozygous deletion of *CFHR1*.

In a second part of the thesis the aim was to describe the clinical and biological features of aHUS patients with anti-FHs present in our cohort.

In the last part, the presence of *HLA-A*, *B*, *C*, *DRB1*, *DRB3*, *DRB4*, *DRB5*, *DQA1*, *DQB1*, *DPA1*, and *DPB1* risk alleles in aHUS patients with anti-FH antibodies compared to healthy controls both carrying the homozygous *CFHR1* deletion was evaluated with the aim to estimate the OR for each risk allele. The final aim was to investigate the affinity of FHR1/FH peptides with the newly identified predisposing MHC molecules using *in silico* assay.

3 Materials and methods

3.1 Patients

Atypical HUS was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematocrit less than 30%, hemoglobin less than 100 g/L, serum lactate dehydrogenase greater than 460 U/L, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, platelets less than 150×10^9/L, negative Coombs test to exclude autoimmune hemolytic anemia, associated with acute renal failure.

Three hundred and five (305) unrelated patients affected by aHUS were recruited through the International Registry of HUS/TTP (Ranica, BG, Italy). Patient data were handled in respect of confidentiality and anonymity. All patients provided informed written consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Azienda Sanitaria Locale, Bergamo, Italy.

Twenty-seven aHUS patients and one patient affected by Dense Deposit Disease have been included in the HLA study.

3.2 Healthy subjects

One thousand three hundred and ninety-nine (1399) Caucasian healthy subjects (1358 were Italians) were used as controls. Samples of healthy subjects were

kindly provided by "Associazione Volontari Italiani Sangue" (AVIS, Milano, Italy) or by Italian Bone Marrow Donors Registry in collaboration with Prof. Amoroso (Azienda Ospedaliera – Universitaria, Città della Salute e della Scienza, Torino, Italy). Healthy subject data were handled in respect of confidentiality and anonymity. All controls provided informed written consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Azienda Sanitaria Locale, Bergamo, Italy.

Sixty-four healthy subjects with the homozygous *CFHR1* deletion were used as "super controls" in HLA study.

3.3 Blood, serum/plasma and DNA samples

Blood samples of patients and healthy subjects were collected on EDTA tubes and stored at –20°C. Patient blood samples were collected during the acute phase or, in most cases, during convalescence. Genomic DNA was isolated from blood using Nucleon BACC2 Genomic DNA Extraction Kit (Illustra), and stored at -20°C.

Nucleon BACC2 Genomic DNA Extraction protocol:

- *4 volumes of Cell Lysis Buffer was added to 6 ml of whole blood;*
- *After 4 min mix incubation at room temperature (RT), centrifugation at 1300g for 5 min;*
- *Cell pellet was resuspended in 2 mL of Reagent B;*
- *Solution was transferred to polypropylene tube and 0.75 µg of RNAse were added; incubation for 30 min at 37°C;*

- *500 µL of $NaClO_4$ solution was added to deproteinize the samples;*
- *After mixing, 2 mL of pre-cooled chloroform was added;*
- *300 µL of Nucleon Resin was added followed by a centrifugation at 1300g for 3 min;*
- *The top phase containing the non-precipitated DNA was moved into a polypropylene tube containing 3 volumes of cold absolute ethanol to precipitate the DNA;*
- *DNA was then removed, air dried and resuspended in 1X TE (100 mM Tris-HCl, 10 mM EDTA, pH 7.5.*

DNA samples from Italian bone marrow donors of Turin were sent already available for genetic analyses.

DNA concentration and quality were determined measuring the absorbance at 260 nm using a UV microvolume spectrophotometer and taken as reference 1X TE Buffer without DNA. The ratios A_{260}/A_{280} and A_{260}/A_{230} were also evaluated to exclude the presence of protein and ethanol/phenol contaminations in the sample.

Serum or plasma EDTA samples were obtained after centrifugation and stored at –80°C. For samples not collect in our clinical centre, sera and/or plasma were sent with dry ice. Serum or plasma EDTA aliquots were available for 305 patients and 111 healthy controls.

3.4 Mutations screening and genotyping

In all aHUS patients with anti-FHs, screening of *CFH*, *CD46, CFI*, *CFB*, *C3,* and *THBD* was performed by direct sequencing (Sanger method). The sequencing also included the *CFH* basal and proximal promoter and *CD46* 3' UTR region.

All primers used in PCR and sequencing reactions were designed using Primer 3 online software 0.4 (http://frodo.wi.mit.edu/primer3/input.htm) to cover all exons and flanking intronic regions and to obtain amplicons ranging from about 300 bp to about 800bp sizes suitable for the direct sequencing performed on 3730 Sequence Analyzer (Life Technologies). In addition, all primer sequences were imputed by looking for similar known sequences using the Blast program available from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to avoid non-specific amplifications.

Ninety-five separate PCR protocols have been optimized in order to avoid non-specific PCR products. Annealing temperatures were established for each amplicon based on the primers melting temperatures. Times for denaturation, annealing and extension steps were established accordingly to amplicon size and GC amplicon composition. Generally, a PCR mix contained: PCR Buffer (10X), dNTPs mix (200 μM), $MgCl_2$ (1.25-2.25 mM), primers (5 μM), Ampli TaqGold DNA Polymerase (0.5 U, Thermo Fisher Scientific), patient/healthy control DNA (30-50 ng), and double-distilled H_2O (ddH_2O) to a final volume of 10 μL. In each PCR a blank sample was included which contained all the reagents with the exception of DNA. PCRs were performed in a thermocycler (2700 or 9700, Applied Biosystem) following specific protocols for each amplicon. A standard

amplification protocol was: initialization at 94°C for 10 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at varying temperature for 30-35 sec, extension at 72°C for 45 sec; final extension at 72° C for 10 min.

Genotyping of the *CFH* SNPs c.1-332C>T (rs3753394), c.184G>A Val62Ile (rs800292), c.1204T>C Tyr402His (rs1061170), c.2016A>G Gln572Gln (rs3753396), c.2808G>T Glu936Asp (rs1065489), *CD46* SNP c.*783T>C (rs7144), *C3* SNPs c.941C>T Arg102Gly (rs1047286), c.304C>G Pro3174Leu (rs2230199), *CFB* SNPs c.94C>T Arg32Trp (rs12614), c.95G>A Arg32Gln (rs641153), and *THBD* SNP c.1418C>T Ala473Val (rs1042579) was performed during the sequencing. The genotyping of *CFH* c.2237-543G>A (rs1410996) was additionally performed by direct sequencing (forward primer, 5'-AATGTGCTGCAGGGTTGGTG-3'; reverse primer, 5'-AGTCACACTGTAAGTGAATCAC-3'). A region starting from c.1-1070 nucleotide including *CFH* distal promoter have been also sequenced (forward primer, 5'-TGCTCACTAACAGGCACA-3', reverse primer, 5' CATTGTCTGGGTGCTGATTG-3').

CFHR1 exons and flanking intronic regions were sequenced in all patients with anti-FHs that did not carry the homozygous deletion of *CFHR1*, as described in Abarrategui-Garrido *et al*.[43]

The PCR success was evaluated by the agarose gel. To this purpose, 3 µL of PCR product were mixed with 1 µL Fermentas 6x Loading Dye (Thermo Fisher Scientific) and 2 µL ddH_2O. The amplified product was electrophoresed at 120

volts for 20-30 min in 1% agarose gel prepared by melting agarose in 1X TBE buffer (Qiagen) in presence of fluorescent nucleic acid dye Gel Red (10,000X, Biotium). After electrophoresis, agarose gel was exposed to UV light and a digital photo was obtained. Finally, the band of the amplified product was compared to the band profile of GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific) which contained control PCR products ranging from 100 bp to 1,000 bp. The PCR product was considered specific if the expected molecular weight of the band corresponded to the observed molecular weight and no other bands were present. Moreover, no amplification bands had to be present in the blank sample.

In order to remove unused primers and deoxynucleotides from amplified PCR products before sequencing reaction, ExoSAP-IT product (USB Corporation, USA) was used.

ExoSAP-IT purification protocol:

- *2 μL of ExoSAP-IT mix was added to 5 μL of PCR product*
- *Incubation at 37°C for 15 min and enzyme inactivation at 80°C for 15 min*

The purified PCR products were sequenced using the BigDye terminator v.3.1 cycle sequencing kit (Life Technologies, USA) and appropriate primers.

BigDye terminator v.3.1 cycle sequencing protocol:

- *BigDye v3.1 Sequencing Buffer (5X)*
- *BigDye Terminator (0.5 μL)*
- *primer (4 μM)*

- *purified PCR product (2 µL)*
- *ddH_2O to a final volume of 10 µL.*

The sequencing reactions were performed in a thermocycler (2700 or 9700, Applied Biosystem) following the subsequent steps: initialization at 96°C for 1 min; 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec; final extension at 60°C for 4 min.

Before loading the sequencing products into the 48-capillary 3730 DNA Analyzer (Life Technologies), the BigDye XTerminator Purification kit was used to sequester salt ions, unincorporated dye terminators and dNTPs to prevent their co-injection with dye-labeled products. To perform the XTerminator purification, 55 µL of BigDye Solution (10 µL of XTerminator Solution and 45 µL of SAM Solution) were added to 10 µL of sequencing product. After vortexing for 30 min and centrifugation at 1500g for 2 min, the purified dye-labeled products were present in the supernatant and were analyzed by the DNA Sequencer using specific BigDye XTerminator run module.

The sequencing results were analyzed with Sequencher Analyzer 5.0 software (Gene Codes Corporation, USA) and comparing each electropherogram to the reference sequences of *CFH*, *CD46, CFI*, *CFB*, *C3, THBD*, and *CFHR1* reported on the Ensembl database (www.ensembl.org).

Genetic variants were defined according to Human Genome Variation Society (HGVS) guidelines. Likely pathogenic variants have been defined as genetic variants with a Minor Allelic Frequency (MAF) reported on ExAc database

≤0.001 or predicted as "probably damaging" by Polyphen 2 software available online (http://genetics.bwh.harvard.edu/pph2). The HumVar was the PolyPhen 2 parameter used to evaluate the effect of rare variations as suggested by the Polyphen 2 software guidelines. I have also considered as pathogenic the variants with a functional effect proved by *in vitro* studies available in literature despite their frequencies could be higher than 0.001.

The expectation maximization algorithm by Haploview software (Broad Institute of MIT and Harvard) was used to estimate *CFH* haplotype frequencies using as minimal informative *CFH* SNPs the rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs1065489, as described by Rodriguez-De Cordoba *et al*.[43, 162]

3.5 Measurement of *CFH*, *CFHR1*, and *CFHR3* copy number

Multiplex-ligation dependent probe amplification (SALSA MLPA P236-A3 ARMD, MCR-Holland, Netherlands) was used to evaluate the presence of copy number variations (CNVs) in *CFH, CFHR3, CFHR1, CFHR2, CFHR5* genes in aHUS patients with anti-FHs. In P236-A3 kit there were 9 probes for *CFH*, 4 probes for intergenic region between *CFH* and *CFHR3*, 5 probes for *CFHR3*, 4 probes for *CFHR1*, 4 probes for *CFHR2*, and 3 probes for *CFHR5* (Table 2.1). In MLPA P236-A3 kit, probe mix also included 8 reference probes which recognized genomic regions for which CNV are absent. For each MLPA experiment, at least 3 DNA from healthy controls with no CN abnormalities and a blank sample with all the reagents without DNA, have been included.

Probe number	Probe Name	Hybridisation Sequence (20 nt adjacent to ligation site)
1	*CFH* exon 1	TGCTACACAA-ATAGCCCATA
2	*CFH* exon 2	GGTCTGACCA-AACATATCCA
3	*CFH* exon 3	TCCTTTTGGT-ACTTTTACCC
4	*CFH* exon 4	ATTACCGTGA-ATGTGACACA
5	*CFH* exon 6	AAAGAGGAGA-TGCTGTATGC
6	*CFH* intron 10	TAGGTAGTCA-TATTTGGAAC
7	*CFH* intron 12	TGGACACATT-ATGATTGAGT
8	*CFH* exon 13	AGTTGGACCT-AATTCCGTTC
9	*CFH* exon 18	GGAACCATTA-ATTCATCCAG
10	CFH exon 19	AGGATGTGTA-TAAGGCGGGG
11	CFH intron 20	GAATTCTATT-TACACTTCCG
12	CFH intron 21	TAATAGGGTA-TATTATTTTC
13	CFH intron 22	GAAAAATCTC-TGTGATGAGT
14	CFH exon 23	AGCTTTATTC-GAGAACAGGT
15	*CFH* intron 23	TCAATACATA-AATGCACCAA
16	*CFH* intron 23	CACTTATACA-TGCAATCCGT
17	*CFH* intron 23	AGTCCGAGGT-AGAAAGGGAC
18	*CFH* intron 23	GTGGTAATCT-TGGCTCTCAG
19	*CFHR3* intron 1	AGGTAAGTTA-AAAGAGATCT
20	*CFHR3* intron 1	CATTTTCTTG-TGGAATTACAGC
21	*CFHR3* intron 3	CGGACGACAG-TCTCAGACTT
22	*CFHR3* intron 4	GGGTTATATG-AATTCCTACA
23	*CFHR3* exon 6	TCCCTTCCCG-ACACACTGCTTG
24	*CFHR1* intron 3	AGAGTTTCAG-GTCCATGTGT
25	*CFHR1* intron 5	AATCTGTGAT-TATTTTGTTA
26	*CFHR1* exon 6	CCTGTTCTCA-AATAAAGCTTCT
27	*CFHR1* exon 6	TTTTCCAAGT-TTTAATATGG
28	*CFHR2* intron 1	TGTCTGTACT-TGGAGTTTCG
29	*CFHR2* intron 2	AGATCATAAA-CACTTGATAA
30	*CFHR2* intron 3	AATACCTGTG-TGTGGTTTATAG
31	*CFHR2* exon 4	ATATGCTCCAGG-TTCATCAGTT
32	*CFHR5* exon 1	TGGGTATCCA-CTGTTGGGGG
33	*CFHR5* exon 2	TGAAGAAGAT-TATAACCCTT
34	*CFHR5* exon 3	CTTCAGGACT-AATACATCTG

Table 3-1. MLPA probes used to determine *CFH* and *CFHR* copy number.

Probes included in SALSA MLPA P236-A3 ARMD in black, probes designed in our laboratory in red.

MLPA protocol:

DNA denaturation

- *5 µL of DNA at 20 ng/µL (100 ng) were denatured at 98°C for 5 min and then cooled at 25°C;*

Hybridization of probes to DNA

- *3 µL of hybridization mix (1.5 µL MLPA buffer and 1.5 µL probe mix) was added;*
- *After mixing, incubation at 95°C for 1 min and at 60°C for 17 hours;*

Ligation of hybridised probes

- *After setting the thermocycler temperature at 54°C, 32 µL of ligase master mix was added (25 µL ddH_2O, 3 µL Ligase Buffer A, 3 µL Ligase Buffer B, and 1uL Ligase-G5 enzyme);*
- *After mixing, incubation at 54°C for 15 min (for ligation) and at 98°C hours for 5 min to inactivate the enzyme;*

PCR amplification of ligated probes

All ligated probes were amplified simultaneously using the same PCR FAM labeled primer pair:

- *10 µL of PCR master mix was added to each tube (7.5 µL ddH_2O, 2 µL SALSA PCR primer mix, and 0.5 µL SALSA polymerase);*

- *After mixing, the tubes were placed in the thermocycler for the PCR amplification. PCR program: 35 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec; final extension at 72°C for 20 min;*
  All this steps was performed in 2700 thermocycler (Applied Biosystem).

Capillary electrophoresis of PCR products

- *Fragment separation was done on the 48-capillary 3730 DNA Analyzer (Life Technologies) using as size marker LIZ GS500 and electrophoresis specifications reported on "MLPA General Protocol" available on www.mlpa.com.*
- *GeneMapper Software (Life Technologies) provided the DNA sizing and quality for each amplified fragment and it was possible to extrapolate the raw data of the peak heights relative to each amplified fragment.*

Result analysis

- *MLPA data analysis was performed with Microsoft Excel. Geometric mean of the peak heights relative to the reference fragments has been used for the intrasample normalization. The ratio between the normalized peak heights of CFH-CFHR fragments of the patient and the normalized peak heights of CFH-CFHR fragments of the healthy control was used for the intersample normalization. Ratios between 0.7 and 1.3 were considered as correspond to 2 copies, ratios between 0.4 and 0.7 meant the presence of 1 copy (heterozygous deletion) and absence of probe signals as correspond to 0 copies (homozygous deletion).*

3.6 Measurement of terminal region of *CFH* copy number

In addition to probes included into P236-A3 kit, 5 home designed probes covering the terminal region of *CFH* are used (showed in red in Table 2.1). For this analysis the SALSA MLPA P300-B1 probemix was used, which contained 14 reference probes for the intrasample normalization. The MLPA protocol adopted was the same used with P236 kit, with the only exception for the hybridization step in which 3 μL of hybridization mix including 1.5 μL of MLPA buffer, 0.5 μL of home designed probe mix, and 1 μL of P300 reference probe mix was added. The home designed probe mix was obtained by combining 0.8 μL of each probe solution (1 μM) in a final volume of 200 μL in 1X TE.

3.7 Measurement of *CFHR4* copy number

The *CFHR4* CN was evaluated by a multiplex PCR (mPCR) as reported by Moore *et al*.[107] The primers were specific for *CFHR4* intron 1, *CFHR4* exon 2, and *CFHR1* intron 3. In addition as suggested in Moore *et al.* specific primers for *KCNT2* exon 9, and *KCNT2* exon 17 were added for the intrasample normalization step. All primers used were labeled with fluorescent 5'FAM.

CFHR4 mPCR protocol

- *150 μg of DNA*
- *Buffer 10X*
- *200 μM dNTPs mix*

- *6.7 mM MgCl2*
- *12.5 pM of each primer*
- *0.5 U Ampli TaqGold DNA Polymerase*
- *ddH_2O to a final volume of 25 µL.*

The PCR was performed in a thermocycler (2700 or 9700, Applied Biosystem) and the PCR program was the following conditions: denaturation at 95°C for 10 min; 20 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec; final extension at 72°C for 20 min. After PCR, the fragment separation was done by capillary electrophoresis on the 48-capillary 3730 DNA Analyzer (Life Technologies) using as size marker LIZ GS500 (Life Technologies) and the same electrophoresis specifications adopted for MLPA analysis. The mean of peak heights relative to *KCNT2* amplified products were used for the intrasample normalization. The ratio between the normalized peak heights of *CFHR4* amplified products relative to the patient and the normalized peak heights of *CFHR4* amplified products relative to the healthy control was used for the intersample normalization. Ratios between 0.7 and 1.3 were considered as correspond to 2 copies, and ratios between 0.4 and 0.7 meant the presence of 1 copy (heterozygous deletion). The analysis of *CFHR1* intron 3 CN by mPCR was used to validate the result making a comparison with the MLPA results.

3.8 Homozygous *CFHR1* deletion evaluation in a large cohort of patients and controls

Multiplex PCR to evaluate homozygous *CFHR1* deletion in a large cohort of aHUS patients and controls was done co-amplifying a 133 bp fragment in intron 3 of *CFHR1* and a 83 bp control fragment in the promoter of *RNAse P*. The primers used were:

CFHR1-For 5'-ATCACTACACATGGACCTGAAA-3',
CFHR1-Rev 5'-GATGTGGAAAAATAAAAGAAAATAAGTC-3',
RNAseP-For 5'-TAGATACCGTGTGCGTGCAT-3',
RNaseP-Rev 5'-GGGGTTCCAATTCCCAACTA-3'.

CFHR1 mPCR protocol

- *70 ng of DNA*
- *Buffer 10X*
- *200 µM dNTPs mix*
- *3 mM MgCl2*
- *0.8 µM of each CFHR1 primer*
- *0.23 µM of each CFHR1 primer*
- *0.5 U Ampli TaqGold DNA Polymerase*
- *ddH_2O to a final volume of 15 µL.*

The PCR was performed in a thermocycler (2700 or 9700, Applied Biosystem) and the PCR program was the following conditions: denaturation at 95°C 10 min; 38 cycles at 95°C 30 sec, 57°C 45 sec, 72°C 30 sec; final extension at 72°C 10

min. PCR products were run on 2.5% agarose gel. Concurrent absence of the *CFHR1* amplicon band and presence of the *RNAseP* band in the agarose gel indicated homozygous *CFHR1* deletion.

3.9 SDS-PAGE and Western blotting

The presence and band profile of FHR1 was studied by Western Blotting in all patients with anti-FHs and with at least one copy of *CFHR1*. Serum samples were diluted 1:40 in loading buffer (4X Laemmil Sample Buffer, Bio-Rad) in absence of reducing conditions and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Mini-Protean TGX Precast Gels, BioRad). Proteins were transferred to polyvinylidene difluoride membrane (PVDF, Trans-Blot Turb Midi PVDF Transfer; Bio-Rad) and blocked with 4% skim milk and 1% bovine serum albumin (BSA). Mouse anti-human FHR1 IgG (kindly given by Prof. Zipfel) at 1:1,000 dilution in blocking solution, which recognized the N-terminal domains of FHR1, has been incubated for 1 hour. The antibody detection was performed after 1 hour incubation with horseradish peroxidase (HRP) goat anti-mouse IgG at 1:5,000 dilution in blocking solution followed by enhanced chemiluminescence (ECL) substrate (Amersham) addition.

3.10 Anti-FH ELISA assay

The presence of anti-FHs was evaluated by a home-made Enzyme-Linked ImmunoSorbent Assay (ELISA) as previously described in Dragon-Durey *et al.* with the addition of some modifications.[98] Microtiter plates (Nunc MaxiSorp,

Denmark) were coated with 0.1 µg (100 µL at 1 µg/mL) of purified human FH (Calbiochem). After overnight incubation at 4°C, the plate was washed 3 times with 200 µL of PBS, 0.1% Tween20, 0.2M NaCl, and blocked with 200 µL of PBS, 0.1% Tween20 and 0.3% milk for 1h at room temperature (RT). After 3 washes with PBS, 0.1% Tween20, 0.2M NaCl, 50 µL of plasma EDTA/serum samples at 1:100 dilution in blocking solution were added in duplicated wells, and incubated for 40 minutes at RT. A parallel plate was set up in absence of FH coating in which only blocking solution was added to evaluate the presence of non-FH specific background. After 4 washes with PBS, 0.1% Tween20, 0.2M NaCl, goat anti-human IgG antibody conjugated with HRP (Sigma-Aldrich) was added and incubated for 1 hour at RT. After 6 washes, 100 µl of tetramethylbenzidine substrate (TMB) were added, and incubated for 2-5 minutes. The reaction was stopped with 100 µL of 10% H_2SO_4, and the optical absorbance was read at 450 nm. For standard curve a positive control kindly gifted by Dr. Dragon-Durey with 2,000 AU/mL titer at 1:100 dilution was used. A positive control and a negative control were present in each experiment. The sample concentrations expressed in arbitrary units/ml (AU/mL) were determined by extrapolation from a sigmoidal curve obtained by Microplate Manager 6 Software (Bio-Rad) and the background derived from wells without FH coating was subtracted. To confirm the antibody positivity, the ELISA was repeated adding 15 µL of FH into each serum/plasma sample to verify whether this excessive amount of FH was able to inhibit the autoantibody binding to FH coated on the plate wells. If the optical absorbance decreased about 50% with the addition of the exogenous FH compared to the optical absorbance obtained with the standard protocol (the diluted sample without the addition of FH), it indicated the presence

of specific autoantibodies against FH antigen. More details about the accuracy of the anti-FH ELISA assay are reported in the third chapter and are an integral part of thesis results.

3.11 Epitope mapping of anti-FHs

Molar equivalent concentrations of amino terminal FH (SCRs 1-5) and C terminal FH (SCRs 15-20) fragments (kindly given by Prof. Peter Zipfel) were immobilized on microtiter plates (Nunc MaxiSorp, Denmark). ELISA assay was performed following the standard protocol used to assess the presence anti-FHs. The results were expressed as absorbance at 450 nm and were obtained from replicated assays.

3.12 FH ELISA assay

FH levels were evaluated by a home-made ELISA method. Microtiter plates (Nunc MaxiSorp, Denmark) were coated with 0.15 μg (100 μL at 1.5 μg /mL) of purified sheep polyclonal anti-human factor H antibody (Abcam). After overnight incubation at 4°C, the plate was washed 3 times with 200 μL of PBS, 0.05% Tween20, and blocked with 200 μL of PBS, 1% BSA for 1h at RT. After 4 washes with PBS, 0.05% Tween20, 100 μL of plasma EDTA/serum samples at 1:10,000 dilution in blocking solution were added in duplicated wells, and incubated for 2 hours at RT. After 4 washes with PBS, 0.05% Tween20, 100 μL of mouse monoclonal anti-human factor H (LifeSpan BioSciences), which recognized the N-terminal domain of FH, were added at 1:10,000 dilution in blocking solution.

The plate was washed 4 times with PBS, 0.05% Tween20 and 100 µL of goat anti-mouse antibody conjugated with HRP (Thermo Fisher) at 2,000 dilution were added and incubated for 1 hour at RT. The plate was washed 4 times with PBS, 0.05% Tween20, and 100 µL of TMB were added. The peroxidase reaction was stopped after 8 minutes with 100 µL of 10% H_2SO_4. The optical absorbance was read at 450 nm. For the first point of the standard curve purified human FH (Calbiochem) at 1:4,000 dilution was used. Healthy control samples were added in each experiment. The sample concentration expressed in mg/mL was determined by extrapolation from a sigmoidal curve obtained by Microplate Manager 6 Software (Bio-Rad). The normal control range was 172-507 mg/L.

3.13 HLA typing

An high resolution sequencing using exon specific primers (SBT, Sequencing-Based Typing) to include all variations coding for the antigen recognition site has been performed in exons 2 and 3 of MHC class I genes (*HLA-A*, *HLA-B,* and *HLA-C*) and in exon 2 of MHC class II genes (*HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4*, *HLA-DRB5*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DPA1*, and *HLA-DPB1*) in 27 aHUS patients with anti-FH autoantibodies (of whom 24 showing the FHR1 deficiency), in 31 Italians healthy controls all carrying the homozygous *CFHR1* deletion, and in 1 patient affected by Dense Deposit Disease carrying anti-FHs and the homozygous *CFHR1* deletion by Histogenetics (Ossining, New York, USA).

Luminex technology for HLA typing by PCR-Sequence Specific Oligonucleotides Probes (SSO) was performed in 33 healthy controls by the Unit of

Immunogenetics and Transplant Biology directed by Prof. Amoroso (Azienda Ospedaliera – Universitaria, Città della Salute e della Scienza, Torino, Italy)

For all patients and the large majority of controls the typing resolution was high enough to allow a four digit HLA nomenclature, according to the indications of World Health Organization Nomenclature Committee for Factors of the HLA system (www.ebi.ac.uk/imgt/hla/stats.html).

3.14 Statistical analyses

All the statistical tests have been done with "MedCalc" software (Version 12.2.1.0 Copyright © 1993-2012). Chi-square test or Fisher's exact test were used to compare the frequency of genotypes and alleles between patients and healthy controls, as appropriate. Findings were considered statistically significant at p-values < 0.05 after Bonferroni correction. Odds ratio (OR) was reported with the 95% confidence interval. ANOVA and Mann-Whitney tests were used to compare the mean and the median of parametric and non-parametric variables, respectively.

Power analysis for the HLA study has been performed with "PS Power and Sample Size Calculations" software **(**Version 3.0, January 2009, Copyright © 1997-2009 by William D. Dupont and Walton D. Plummer). *HLA* allele and carrier frequencies were compared by Chi-square test or by Fisher's exact test when the number of subjects in the 2 X 2 contingent tables was five or less. Bonferroni correction was applied to p-values considering the number of tested alleles at each HLA gene. Findings were considered statistically significant at p-values < 0.05 after correction. OR was calculated only for risk alleles and given with the 95% confidence interval.

4 Variants in complement genes associated with anti-factor H autoantibodies in atypical Hemolytic Uremic Syndrome (aHUS)

4.1 Introduction

Atypical hemolytic uremic syndrome (aHUS) is a rare kidney disease with a prevalence of 1:100,000 and characterized by thrombotic microangiopathy and acute renal failure.[57] It is mainly caused by dysregulation or hyperactivation of the alternative pathway (AP) of complement system and more rarely by defects in diacylglycerol kinase ε.[57, 62, 100] About half of patients are carriers of mutations in genes encoding AP regulatory proteins (such as factor H, factor I, membrane cofactor protein CD46, and thrombomodulin) or in genes enconding C3 and factor B that form the AP C3 and C5 convertases, C3bBb and $(C3b)_2Bb$.[62] Autoantibodies against factor H (anti-FHs) have been also reported in 5-14% of patients in European cohorts and up to 50% of aHUS cases in South Asia.[98, 105-107, 110] Anti-FHs bind multiple FH epitopes mainly located at the C-terminal domain which is often the hot spot of genetic alterations in aHUS.[115, 121] The presence of anti-FHs determines the impairment of factor H functions compromising the self-surface protection from complement activation and the development of aHUS.[115]

Anti-FHs are strongly associated with the deficiency of factor H Related 1 (FHR1).[107, 123] FHR1 is encoded by the *CFHR1* gene that is located in the regulator of complement activation (RCA) gene cluster on chromosome 1q32

together with *CFH* and other 4 factor H related genes (*CFHR*2-3-4-5). The high level of sequence identity among *CFH* and *CFHR* genes can favor genomic rearrangements through non allelic homologous recombination (NAHR). FHR1 deficiency is most often caused by a polymorphic homozygous deletion of *CFHR3* and *CFHR1*. The frequency of the homozygous deletion of *CFHR3* and *CFHR1* is 5% in European, 9% in Asian, and about 20% in sub-Saharian African populations.[42, 110, 163] It appears clear that a common factor such as FHR1 deficiency is not sufficient to cause the development of a rare disease like aHUS.

In literature other defects in genes encoding complement proteins have been reported in about 15% of aHUS patients with anti-FHs but a correlation with the generation of FH autoantibodies has not been reported.[43, 107, 128] Common variants in *CFH* and *MCP* (SNPs and haplotypes) have been reported to increase the risk of developing aHUS in subjects also carrying mutations in complement genes.[86, 87] Interestingly, in Moore *et al.* report of all 13 aHUS patients who were positive for anti-FHs carried the *CFH* "A" variant at the rs3753396 and "G" variant at rs1065489 suggesting a possible role of particular *CFH* haplotypes in predisposing to development of anti-FHs.[107] Of note, the common variants in FH and in the AP C3 convertase proteins, FB and C3, have been also reported to be associated with other complement mediated diseases, such as age-related macular degeneration (AMD) and Dense Deposit Disease (DDD).[164-167] Also *THBD* c.1418C (Ala473) common variant is described to increase the risk to develop C3 glomerulopathy.[55]

In this chapter of the thesis: 1) I report the results of the screening of 305 unrelated patients affected by aHUS; 2) I identify a group of 30 patients with anti-

FHs; 3) I document the association between the FHR1 deficiency and anti-FHs in our cohort of aHUS patients; 4) I focus on rare and common genetic variants in complement genes that can predispose to the development of aHUS in patients with anti-FHs. In these analyses "super controls", *i.e.* healthy subjects carrying the homozygous deletion of *CFHR1*, were used as reference group.

4.2 Specific aims

To set up an ELISA to specifically detect anti-FHs in plasma/serum samples

To identify true and false positive samples for anti-FHs.

To determine the prevalence of anti-FHs in our cohort of patients with aHUS.

To study which FH epitopes are recognized by anti-FHs in our patients and to evaluate whether a correlation exists between the FHR1 deficiency and the anti-FH recognition site.

To statistically evaluate the association between homozygous deletion of *CFHR1* and the development of anti-FH-mediated aHUS.

To evaluate whether genetic mutations in complement genes were associated with anti-FH - mediated aHUS.

To evaluate whether common variants in complement genes were associated with anti-FH - mediated aHUS using as reference group “super controls”, *i.e.* healthy subjects with the homozygous deletion of *CFHR1*.

4.3 Results

4.3.1 Assessment of anti-factor H antibodies by ELISA.

An enzyme-linked immunosorbent assay (ELISA) was firstly performed in sera (or plasma EDTA) of healthy controls to set up the assay positivity threshold. Since anti-FH-mediated aHUS has been previously associated to the homozygous deletion of *CFHR1*, 98 Italian blood healthy donors over 40 years of age with 1 or 2 copies of *CFHR1* by MLPA were selected as reference negative control group to define the lower limit of the auto-antibody positivity (Figure 4-1). The mean antibody titer (± standard deviation, SD) assessed by ELISA in the control group was 8.0 AU/mL (±23.8). The threshold positivity has been set at the mean +5SD of individual values recorded in the negative control group (127 AU/mL, Figure 4-1). A less stringent threshold at the mean+2SD (56 AU/mL) of the negative control group was also adopted (Figure 4-1). Samples with anti-FH titer <56 AU/ml were considered as true negative, samples with antibody titer in the range 56-127 AU/mL as possibly positive, and samples with antibody titer >127 as positive.

The presence of anti-FHs was assessed by ELISA in 305 consecutive aHUS patients of the International Registry for whom serum or plasma was available. Forty-one patients showed an antibody titer higher than 56 AU/mL. Twenty-six patients had an anti-FH titer higher than 127 AU/mL and fifteen patients between 56-127 AU/mL.

4.3.2 Assessment of the accuracy of the anti-FH antibody assay

To exclude false positive results, all the samples were also analyzed using wells in which FH was not coated. Since anti-FHs should bind to FH-coated wells but not to those not coated with FH, in true positive samples a significant reduction of the absorbance in the non-FH-coated compared to the FH-coated wells was expected. On the contrary, absence of absorbance reduction in non-FH-coated wells suggested an aspecific signal. A reduction of at least 50% was observed in 30 out of 41 patients with anti-FH titer ≥ 56 AU/mL. To confirm these data the ELISA was repeated in all 41 patients with a titer ≥56 AU/mL adding 15 μg of FH into each serum/plasma sample which is 150-fold the amount of FH used to coat each well. The excessive amount of FH is expected to compete with the FH immobilized on the plate for the binding to the autoantibodies. An absorbance decrease of about 50% in samples added with exogenous FH, indicated that the antibodies were FH specific. Thirty out of 41 patients with a ≥56 AU/mL titer showed a significant reduction of the absorbance in the presence of an excess of FH and this confirmed the data obtained with non-FH-coated wells. This control step had highlighted that the percentage of false positive (11/41) was higher in the possibly positive group (8/15, 53.3%) compared to the likely positive group (3/26, 11.5%).

Overall, 30 out of 305 aHUS patients were found true positive for anti-FHs by ELISA suggesting a prevalence of 9.8% in our aHUS cohort (Figure 4-1). Noteworthy all 11 false positive samples did not show homozygous deletion of *CFHR1* gene that is usually associated with anti-FH development further confirming the specificity of the results.

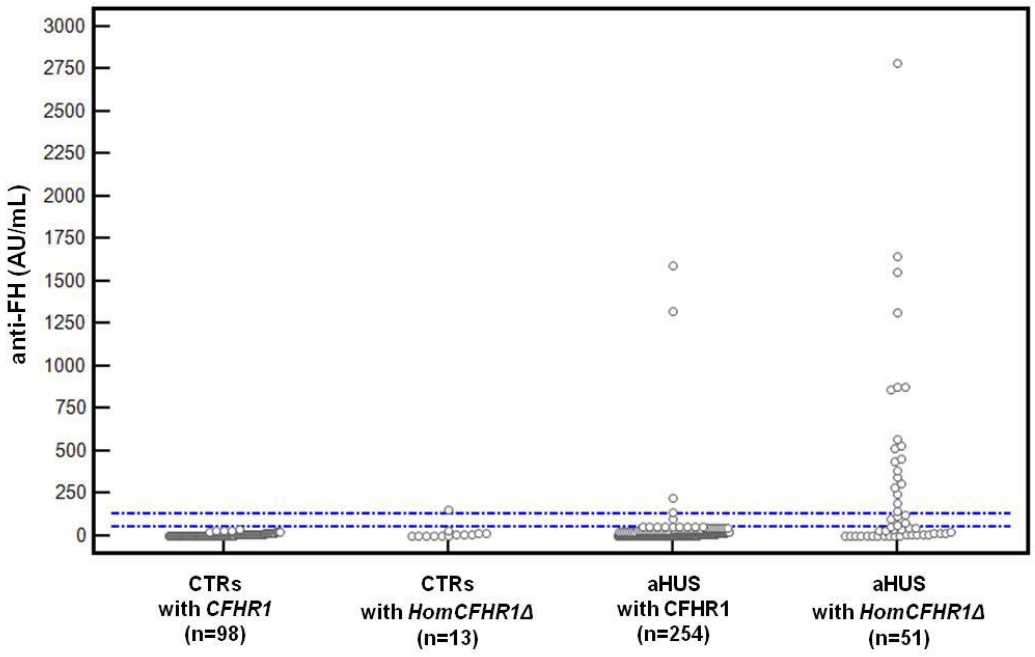

Figure 4-1. Anti-FHs in healthy controls and aHUS patients.

111 healthy controls have been analyzed for anti-FHs through ELISA: 98 were carriers of 1 or 2 copies of *CFHR1* and 13 had 0 copies of *CFHR1* (*HomCFHR1*Δ). 305 aHUS patients have been also analyzed for anti-FHs: 254 were carriers of 1 or 2 copies of *CFHR1*, and 51 had 0 copies of *CFHR1* (*HomCFHR1Δ*). An aHUS patient with FHR1 deficiency (number 23, see Table 3) has been also included in the group of patients defined in this figure as "aHUS with *HomCFHR1Δ*"). For subjects with an anti-FH antibody titer ≥56 AU/mL, it was reported the anti-FH antibody titer obtained from wells coated with FH after the background subtraction obtained in wells without FH coating. The blue lines indicated anti-FH antibody titer mean +2SD (56 AU/mL) and +5SD (127 AU/mL).

4.3.3 Presence of anti-FHs in healthy subjects

Among 98 healthy controls with *CFHR1*, one subject had an anti-FH titer of 229 AU/ml, but in FH-non-coated wells and in the presence of FH excess a significant reduction of the absorbance was not detected indicating that it was a false positive sample.

Plasma samples of 13 healthy controls with 0 copies of *CFHR1* have also been collected and one of them showed anti-FHs with a titer of 193 AU/mL (Figure 4-1). The antibody titer decreased around 50% in both FH-non-coated wells and in the presence of FH excess, meaning that the antibodies were specific for FH and that the sample is a true positive.

Overall, 1 out of 111 controls showed anti-FHs suggesting a prevalence of 0.9% in the healthy population.

4.3.4 Binding of autoantibodies to FH fragments

To discriminate which FH epitopes were recognized by anti-FHs and if there was a correlation between absence of FHR1 and antibody recognition site, ELISA was repeated using wells coated with N terminal FH (SCRs 1-5) and C terminal FH (SCRs 15-20) fragments (kindly given by Prof. Peter Zipfel) at the same molarity used in FH coated wells during the standard procedure to detect anti-FHs. The absorbance of samples from 5 aHUS patients without the homozygous deletion of *CFHR1* (numbers 19, 13, 30, 21, and 26) and from 7 aHUS patients carrying the homozygous *CFHR1* deletion (numbers 16, 14, 15, 10, 25, 18, and 8) are shown in Figure 4-2. In 3 patients without homozygous deletion of *CFHR1* (numbers 19, 13, and 30) and in 4 patients carrying the homozygous deletion of *CFHR1* (numbers 16, 14, 15, and 10), the absorbance was very high when the coating was performed with C terminal FH and very low in presence of N terminal coated FH. In 3 patients with the homozygous deletion of *CFHR1* (numbers 25, 18, and 8) the signal was less intense but higher in presence of FH C terminus than when the coating was done using the N terminal FH fragment. In one patient without the homozygous *CFHR1* deletion (number 21) the absorbance on FH SCRs 15-20 and

on FH SCRs1-5 showed similar values of low intensity in both conditions. In another patient without the homozygous deletion of *CFHR1* (number 26) the anti-FHs did not recognize either C terminal FH or N terminal FH fragments.

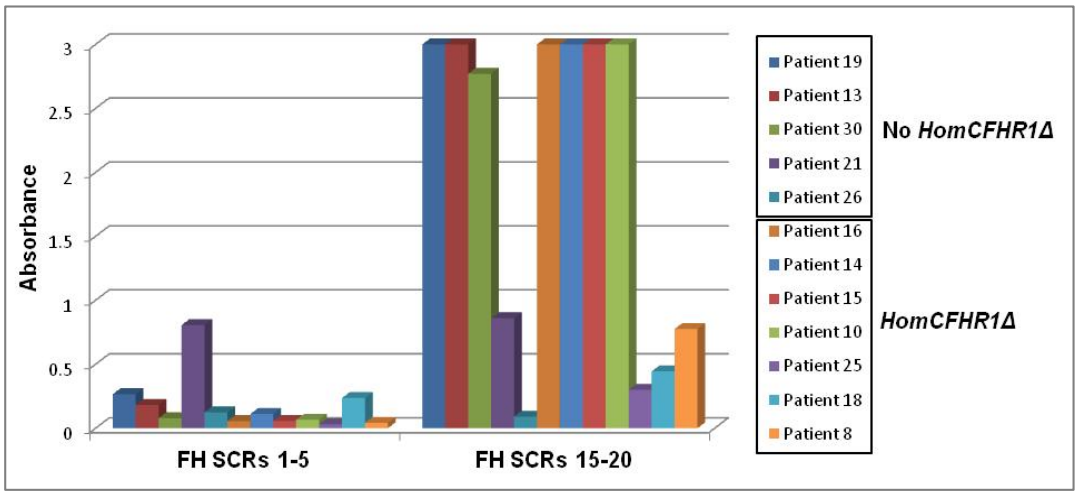

Figure 4-2. Binding site localization of anti-FHs detected in aHUS patients.

Anti-FH binding to FH N terminal fragment (SCRs 1-5) and FH C terminal fragment (SCRs 15-20) evaluated in 12 aHUS patients. Seven patients were carriers of the homozygous *CFHR1* deletion (*HomCFHR1Δ*) while 5 patients presented at least one copy of *CFHR1* (No *CFHR1Δ*). The absorbance was shown on the ordinate axis and it was the result from replicated assays.

4.3.5 Copy number evaluation in *CFHR1, CFHR3* and *CFHR4*

The homozygous deletion of *CFHR1* was evaluated by multiplexing PCRs of an amplicon laying in *CFHR1* intron 3 and of a control amplicon in *RNAseP* gene as decribed in "Materials and Methods". Concurrent absence of the *CFHR1* amplicon band and presence of the *RNAseP* band indicated homozygous deletion of *CFHR1*. The power of this multiplex PCR to identify homozygous *CFHR1* deletion was evaluated in 175 subjects already analyzed by MLPA and sensitivity and specificity were 100% (Table 4-1).

mPCR	MLPA			
	Deleted	Not deleted	Sensitivity (%)	Specificity (%)
Deleted	39	0	100	100
Not deleted	0	139		

Table 4-1. Sensitivity and specificity of multiplex PCR to detect homozygous deletion of *CFHR1* in 175 subjects analyzed by MLPA.

The multiplex PCR was adopted to investigate the presence of homozygous deletion of *CFHR1* in all 305 unrelated aHUS analyzed for anti-FHs and in a large cohort of healthy controls (1399). Fifty aHUS patients (16.4%) and 71 controls (5.1%) resulted homozygous for *CFHR1* deletion (OR [95%CI] = 3.67 [2.493-5.394], p-value = 7.364×10^{-12}, Table 4-2). This difference was enhanced when we focused on aHUS patients with anti-FHs (24/30, 80%) *versus* healthy controls (71/1399, 5.1%; OR [95%CI] = 74.817 [26.64-188.853], p-value = 3.973×10^{-57}, Table 4-2).

Of note, the homozygous deletion of *CFHR1* was significantly overrepresented in aHUS patients resulted positive for anti-FHs compared to aHUS patients negative for anti-FHs (OR [95%CI] = 38.308 [14.354-102.23], p-value = 4.88×10^{-22}, Table 4-2). Interestingly, the homozygous deletion of *CFHR1* was also more abundant in aHUS patients negative for anti-FHs (9.5%) compared to healthy controls (OR [95%CI] = 1.953 [1.222-3.1222], p-value = 0.007, Table 4-2).

	Hom *CFHR1* Δ	No Hom *CFHR1* Δ	OR [95% CI]	p-value
aHUS (n=305)	50 (16.4%)	255 (83.6%)	3.67 [2.493-5.394]	7.364×10^{-12}
Controls (n=1399)	71 (5.1%)	1328 (95%)		
aHUS anti-FH positive (n=30)	24 (80%)	6 (20%)	74.817 [26.64-188.853]	3.973×10^{-57}
Controls (n=1399)	71 (5.1%)	1328 (95%)		
aHUS anti-FH positive (n=30)	24 (80%)	6 (20%)	38.308 [14.354-102.23]	4.88×10^{-22}
aHUS anti-FH negative (n=275)	26 (9.5%)	249 (90.5%)		
aHUS anti-FH negative (n=275)	26 (9.5%)	249 (90.5%)	1.953 [1.222-3.122]	0.007
Controls (n=1399)	71 (5.1%)	1328 (95%)		

Table 4-2. Homozygous deletion of *CFHR1* in aHUS patients (n=305) and healthy controls (n=1399).

Copy number analysis of *CFHR3* and *CFHR1* by MLPA and of *CFHR4* by multiplex PCR was performed in all 30 patients with anti-FH autoantibodies (Table 4-3). I found that the homozygous deletion of *CFHR1* previously detected in 24 out of 30 patients with anti-FHs by multiplex PCR, was caused by the homozygous deletion of *CFHR3* and *CFHR1* in 20 patients (66.7%) and by the heterozygous deletion of *CFHR3* and *CFHR1* combined with the heterozygous deletion of *CFHR1* and *CFHR4* in 4 patients (13.3%). Four patients (13.3%) showed the heterozygous deletion of *CFHR3* and *CFHR1* and two patients (6.7%) no copy number variations in *CFHR* genes (Table 4-3).

Patient anti-FH Ab positive	*CFH* c.1-332C>T (rs3753394)	*CFH* c.184G>A Val62Ile (rs800292)	*CFH* c.1204T>C Tyr402His (rs1061170)	*CFH* c.2016A>G Gln572Gln (rs3753396)	*CFH* c.2237-543G>A (rs1410996)	*CFH* c.2808G>T Glu936Asp (rs1065489	*ΔCFHR3-CFHR1*	*ΔCFHR1-CFHR4*	*CD46* c.*783T>C (rs7144)
1	C T	G G	T T	A A	A A	G G	d d	D D	T T
2	T T	G G	T T	A A	A A	G G	d d	D D	T T
3	C T	G G	T T	A A	A A	G G	d d	D D	T T
4	C T	G G	C T	A A	G A	G G	D d	D d	T T
5	C T	G G	T T	A A	A A	G G	d d	D D	T C
6	C C	G G	T T	A A	A A	G G	d d	D D	T C
7	C C	G G	T T	A A	A A	G G	d d	D D	T T
8	C T	G G	T T	A A	A A	G G	d d	D D	T C
9	C T	G G	T T	A A	A A	G G	d d	D D	T C
10	C C	G G	T T	A A	A A	G G	d d	D D	C C
11	C C	G G	T T	A A	A A	G G	d d	D D	T T
12	C T	G A	T T	A A	A A	G G	d d	D D	C C
13	C C	G G	C T	A A	G A	G G	D d	D D	C C
14	C C	G G	T T	A A	A A	G G	d d	D D	T T
15	C T	G G	T T	A A	A A	G G	d d	D D	C C
16	C T	G G	T T	A G	G A	G T	D d	D d	C C
17	T T	G G	T T	A A	A A	G G	d d	D D	T C
18	T T	G G	T T	A A	A A	G G	d d	D D	T T
19	T T	G G	T T	A G	G A	G T	D d	D D	T T
20	C C	G G	T T	A A	A A	G G	d d	D D	T C
21	T T	G G	T T	G G	G G	T T	D D	D D	C C
22	C T	G A	T T	A A	A A	G G	d d	D D	T C
23*	C T	G A	T T	A G	G A	G T	D d	D D	T C
24	C C	G A	T T	A A	A A	G G	d d	D D	T C
25	T T	G G	T T	A A	A A	G G	d d	D D	T C
26	C C	G A	C T	A A	G A	G G	D d	D D	T T
27	C T	G A	T T	A A	A A	G G	d d	D D	T T
28	C T	G G	C T	A A	G A	G G	D d	D d	T C
29	T T	G G	na	A G	na	G T	D d	D d	T T
30	C T	G A	C T	A A	na	G G	D D	D D	C C

Table 4-3. Detection of *CFH* and *CD46* SNPs and *CFHR3-CFHR1* and *CFHR1-CFHR4* deletions in 30 aHUS patients with anti-FHs.

Genotypes of *CFH* SNPs (rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs106548), *CD46* SNP rs7144, *CFHR3-CFHR1* deletion carrying alleles (D = wild type allele, d = allele with deletion), and *CFHR1-CFHR4* deletion carrying alleles (D = wild type allele, d = allele with deletion). * patient 23 showed the heterozygous *CFHR1* c.104delAfsX, p.D35fsX36. na = not available.

4.3.6 *CFHR1* sequencing

To identify additional patients with FHR1 deficiency, the direct sequencing of *CFHR1* gene was performed in the six patients with anti-FHs that carried at least one copy of *CFHR1* gene. In patient 23 who showed the heterozygous *CFHR1* deletion I also found a frameshift mutation in *CFHR1* exon 2 (c.104delAfsX) that resulted in a truncated protein (p.D35fsX36, Table 3). Thus, overall in our cohort

of patients with anti-FHs - mediated aHUS, 25 out of 30 patients presented a complete FHR1 deficiency (83.3%).

I have also evaluated the presence of the two different FHR1 isoforms (acidic and basic) in patients with anti-FHs. This difference is caused by the nucleotide changes c.469C>T, c.475C>G, and c.523G>C that cause the amino acid changes from 157His, 159Val, and 175Gln in SCR3 domain (FHR1 basic isoform) to 157Tyr, 159Leu, and 175Glu (FHR1 acidic isoform). Of note, the SCR3 of the FHR1 basic isoform is identical to SCR18 of FH. One patient with the heterozygous *CFHR3-CFHR1* deletion showed the basic isoform, two patients with the heterozygous *CFHR3-CFHR1* deletion showed the acidic isoform and two patients with two copies of *CFHR3* and *CFHR1* showed both *CFHR1* isoforms (data not shown). Thus, both acidic and basic isoforms were present in our aHUS patients with anti-FH autoantibodies.

4.3.7 Detection of FHR1 in patient sera

To verify whether the 5 patients who did not show FHR1 deficiency in the above analyses had normal FHR1 in their circulation, I next evaluated by Western blotting, the presence of FHR1 protein in sera of aHUS patients with anti-FHs with at least one copy of *CFHR1* (Figure 4-3). Patient numbers 13, 19, 26 (all with one copy of *CFHR1*) and patient numbers 21 and 30 (with two *CFHR1* copies) showed the FHR1 bands. The patient number 23, who had one copy of *CFHR1* with a frameshift mutation in exon 2 (c.104delAfsX) did not show any FHR1 bands, as expected.

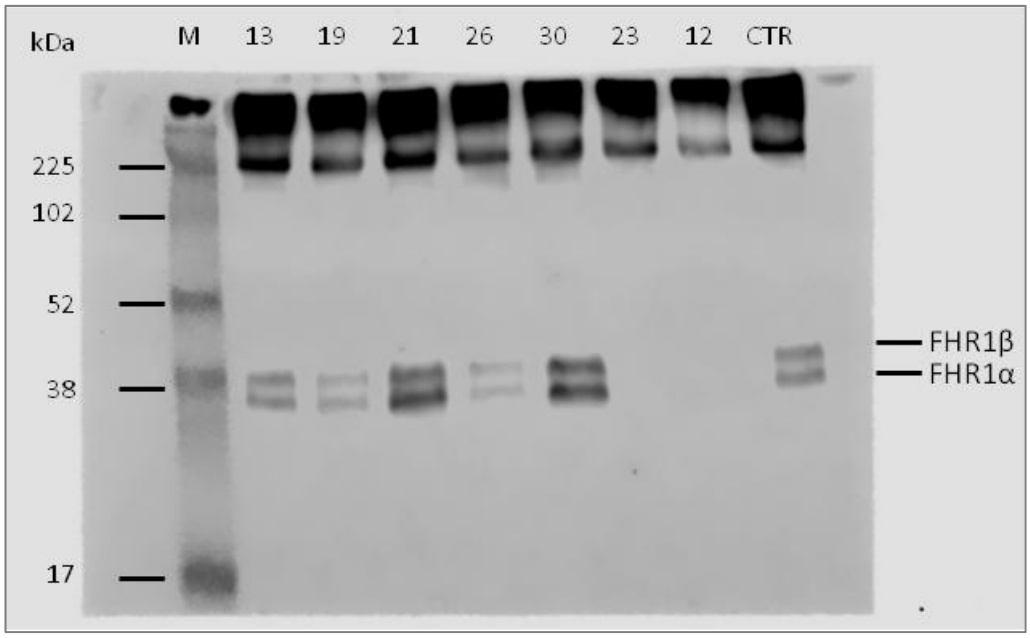

Figure 4-3. Detection of FHR1 by Western blotting in aHUS patients with anti-FHs and at least one copy of *CFHR1*.

Patients number 13, 19, 26 had 1 copy of *CFHR1*, patients 21 and 30 had 2 copies of *CFHR1*, patient number 23 had 1 copy of *CFHR1* with a frameshift mutation in exon 2 (c.104delAfsX), patient 12 had 0 copies of *CFHR1*, and "CTR" was an healthy control with 2 copies of *CFHR1* by MLPA.

4.3.8 Mutations in complement genes in patients with aHUS and anti-FHs

The direct sequencing of *CFH*, *CD46*, *CFI*, *CFB*, *C3*, and *THBD* was performed in all 30 patients with anti-FHs. I found mutations in 11 patients (37%, Table 4-4).

Three patients carried mutations in *CFH* (10%). One Italian patient showed the heterozygous c.2850G>T, p.Gln950His, (rs149474608, ExAc allele frequency = 0.004) that was previously described in one aHUS patient with anti-FHs, in other aHUS patients without anti-FHs, in a patient who have developed TMA after renal transplantation, and also in healthy individuals.[84, 107, 168-171] Another Italian

patient showed the c.2776C>T, p.Cys926Arg and in a Jewish patient I found the heterozygous c.2758T>C, p.Trp920Arg. For both p.Cys926Arg and p.Trp920Arg, frequency data were not available and these variants were not reported in other aHUS cases in literature. Interestingly, p.Cys926Arg and p.Trp920Arg are located in FH SCR15 and p.Gln950His in FH SCR16 which are all central domains of FH molecule without a clear function. *In silico* analyses using Polyphen2 software have predicted all these *CFH* mutations as probably damaging.

In other 3 patients I observed heterozygous mutations in *CD46* (10%). The c.1148C>T, p.Thr383Ile (ExAc allele frequency = 0.0006), was found in an Italian patient and it was previously described in an aHUS family and in a woman affected by idiopathic, spontaneous pregnancy loss.[172, 173] The c.38C>T, p.Ser13Phe (ExAc allele frequency = 0.0001) was found in a Japanese patient also carrying a nonsense mutation in *CFHR1* and it was previously reported in HELLP syndrome and in two Chinese aHUS patients.[174, 175] Finally, I also found a novel mutation in a patient from Turkey, c.762delT, p.Leu254fsX43 that was not reported in ExAc database.

Two patients showed heterozygous mutations in *C3* gene (6.7%). The *C3* c.1774C>T, p.Arg592Trp (ExAc allele frequency = 8.2×10^{-6}) was detected in an Argentine patient who did not carry the homozygous *CFHR1* deletion. This mutation was described in literature as p.Arg570Trp in a patient with aHUS and functional studies demonstrated that it impairs the CD46 binding resulting in a C3b resistant to the cleavage mediated by factor I.[83] Another *C3* mutation was the c.1909G>C, p.Gly637Arg (ExAc allele frequency = 0.0002). It was not reported

in published data and was predicted probably damaging. Both these mutations are localized at the end of the C3 beta chain in the linker domain.

Lastly, 3 patients with anti-FHs were also carriers of *THBD* mutations (10%) that were previously reported in Delvaeye *et al.* (c.1693G>T- p.Asp486Tyr, c.1502C>T - p.Pro501Leu, and c. 241G>A - p.Val81Ile). Functional studies have shown that these variants were less effective in enhancing factor I mediated conversion of C3b to iC3b.[31]

ID patient	Gender	Ethnicity	Popolation	Likely pathogenic variants	MAF	Prediction by PolyPhen	Ref
1	F	Caucasian (Eu)	Italian	nd			
2	M	Caucasian (Eu)	Italian	*CFH* c.2850G>T, p.Gln950His	0.004	Prob D	107, 168-171
3	F	Caucasian (Eu)	Portuguese	nd			
4	F	Caucasian (Eu)	German	nd			
5	M	Caucasian (Eu)	Italian	nd			
6	M	Caucasian (Eu)	Italian	*C3* c.1909G>C, p.Gly637Arg	na	Prob D	
7	M	Caucasian (Eu)	Italian	*THBD* c.1693 G>T, p.Asp486Tyr	0.006	B	31
8	M	Caucasian (USA)	American	nd			
9	F	Caucasian (Eu)	Serbian	nd			
10	F	Caucasian (Eu)	Serbian	nd			
11	F	Caucasian (Eu)	Italian	nd			
12	M	Caucasian (Eu)	Italian	nd			
13	F	Caucasian (Eu)	Turkish	*CD46* c.762delT, p.Leu254fsX43	na	Prob D	nd
14	F	Caucasian (Eu)	Bulgarian	nd			
15	M	Caucasian (Eu)	Polish	*CD46* c.1148C>T, p.Thr383Ile	6×10^{-4}	B	172-173
16	F	Caucasian (USA)	American	nd			
17	F	Caucasian (USA)	American	nd			
18	M	Caucasian (Eu)	Italian	nd			
19	F	Caucasian (Eu)	Italian	*THBD* c.1502C>T, p.Pro501Leu	0.002	B	31
20	F	Caucasian (Eu)	Dutch	nd			
21	F	Hispanic	Argentine	*C3* c.1774C>T, p.Arg592Trp	8×10^{-6}	Prob D	83
22	M	Caucasian (Eu)	Polish	nd			
23*	M	Asian	Japanese	*CD46* c.38C>T, p.Ser13Phe	0.0001	Prob D	174-175
24	M	Jewish	Israeli	*CFH* c.2758T>C, p.Trp920Arg	na	Prob D	nd
25	M	Persian	Iranian	nd			
26	M	Hispanic	Argentine	nd			
27	M	Caucasian (Eu)	Italian	*CFH* c.2776T>C, p.Cys926Arg	na	Prob D	
28	F	Caucasian (Eu)	Italian	nd			
29	M	African-Arab	Yemenian	*THBD* c. 241G>A, p.Val81Ile	9×10^{-6}	B	31
30	M	Caucasian (Eu)	Belorussian	nd			

Table 4-4. Likely pathogenic variants in *CFH*, *CD46*, *C3* and *THBD* genes observed in aHUS patients with anti-FHs.

It was also indicated the ethnicity, the population to which the patient belonged, the allelic frequency from ExAC database, the PolyPhen prediction ("Prob D" meant Probabily Damaging), and the literature references (Ref). nd = not detected

4.3.9 Susceptibility genetic variants

I also investigated whether common genetic variants in *CFH* and *CD46* previously associated with aHUS can promote susceptibility to anti-FH mediated aHUS. The prevalence of *CFH* c.1-332C>T (rs3753394), c.184G>A p.Val62Ile (rs800292), c.1204T>C p.Tyr402His (rs1061170), c.2016A>G Gln572Gln (rs3753396), c.2237-543G>A (rs1410996), c.2808G>T Glu936Asp (rs106548), and *CD46* c.*783T>C (rs7144) was evaluated in all 30 aHUS patients with anti-FHs (Table 4-3) and in 50 "super controls", subjects showing the homozygous deletion of *CFHR1* (Table 4-5). The comparison was also performed between the patients with both anti-FHs and the homozygous *CFHR1* deletion (n = 24) and super controls. I observed that the *CFH* c.2016G (572Gln), c.2237-543G, and c.2808T (936Asp) alleles were overrepresented in all anti-FH positive aHUS patients compared to super controls with the homozygous *CFHR1* deletion (Fisher's exact test, c.2016G: p-value = 0.012, c.2237-543G: p-value= 0.009, and c.2808T: p-value=0.016, respectively, Table 4-6). The allelic frequencies did not show any differences between cases and controls if I considered the patient group with homozygous deletion of *CFHR1* (Table 4-6). Considering that the significance cut-off after the Bonferroni correction was set at 0.007, the significance was lost for all these SNPs. No significant association was found considering the other common *CFH* and *CD46* SNPs (Table 4-6).

Controls with Hom *CFHR1* Δ	*CFH* c.1-332C>T (rs3753394)	*CFH* c.184G>A Val62Ile (rs800292)	*CFH* c.1204T>C Tyr402His (rs1061170)	*CFH* c.2016A>G Gln572Gln (rs3753396)	*CFH* c.2237-543G>A (rs1410996)	*CFH* c.2808G>T Glu936Asp (rs1065489)	*CD46* c.*783T>C (rs7144)
1	C T	G G	T T	A A	A A	G G	T T
2	C T	G A	T T	A A	A A	G G	T T
3	C T	G G	T T	A A	A A	G G	T T
4	C C	G G	T T	A A	A A	G G	T T
5	C T	G G	T T	A A	A A	G G	C C
6	C C	G A	T T	A A	A A	G G	T T
7	C T	G G	T T	A A	A A	G G	T T
8	na	G G	T T	A A	A A	G G	na
9	C T	G G	T T	A A	A A	G G	C C
10	C T	G G	T T	A A	A A	G G	T C
11	C T	G G	T T	A A	A A	G G	T C
12	T T	G G	T T	A A	A A	G G	C C
13	C C	G G	T C	na	G A	G G	T C
14	C T	G G	T T	A A	A A	G G	na
15	C C	G G	T T	A A	A A	G G	T C
16	T T	G G	T T	A A	A A	G G	T C
17	C T	G G	T T	A A	A A	G G	T C
18	C T	G G	T T	A A	A A	G G	T C
19	C T	G G	T T	A A	A A	G G	T T
20	C C	G G	T T	A A	A A	G G	T C
21	C C	G G	T T	A A	A A	G G	T C
22	C C	G G	T T	A A	A A	G G	T T
23	C C	G G	T T	A A	A A	G G	T T
24	na	G A	T T	A A	A A	G G	T C
25	C T	G G	T T	A A	A A	G G	T C
26	C C	G G	T C	A A	G A	G G	C C
27	C C	G G	T T	A A	A A	G G	T C
28	na	G G	T T	A A	A A	G G	T C
29	na	G A	T T	A A	A A	G G	T C
30	na	G G	T T	A A	A A	G G	T C
31	na	G G	T T	A A	A A	G G	T C
32	C C	G G	T T	A A	A A	G G	T C
33	C T	G G	T T	A A	A A	G G	T T
34	C C	G G	T T	A A	A A	G G	T C
35	C T	G G	T T	A A	na	G G	T T
36	T T	G G	T T	A A	A A	G G	T C
37	C T	G G	T T	A A	A A	G G	na
38	T T	G G	T T	A A	A A	G G	T T
39	C T	G G	T T	A A	A A	G G	T C
40	C C	G G	T T	A A	A A	G G	T T
41	C T	G G	T T	A A	A A	G G	T T
42	T T	G G	T T	A A	A A	G G	T C
43	C T	G G	T T	A A	A A	G G	T T
44	C T	G G	T T	A G	G A	G T	T T
45	C C	G G	T T	A A	A A	G G	T C
46	C T	G G	T T	A A	A A	G G	T C
47	C C	G G	T T	A A	A A	G G	T C
48	C T	G A	T T	A A	A A	G G	T T
49	C T	G G	T T	A A	A A	G G	C C
50	na	G A	T T	A A	A A	G G	T T

Table 4-5. *CFH* and *CD46* SNPs in super controls.

Genotypes of *CFH* SNPs (rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs106548) and *CD46* SNP rs7144 in 50 healthy controls with the homozygous deletion of *CFHR1* (super controls). na = data not available

		MM	Freq MM	Mm	Freq Mm	mm	Freq mm	p-value	M	Freq M	m	Freq m	p-value
CFH c.1-332C>T (rs3753394)	aHUS with anti-FH (n=30)	9	0.3	14	0.47	7	0.23	0.414	32	0.53	28	0.47	0.709
	aHUS with anti-FH and Hom*CFHR1* Δ (n=24)	7	0.29	12	0.5	5	0.21	0.587	40	0.54	22	0.46	0.983
	ctr with Hom*CFHR1* Δ (n=43)	15	0.35	23	0.53	5	0.12		53	0.62	33	0.38	
CFH c.184G>A Val62Ile (rs800292)	aHUS with anti-FH (n=30)	23	0.77	7	0.23	0	0.00	0.596	53	0.88	7	0.12	0.238*
	aHUS with anti-FH and Hom*CFHR1* Δ(n=24)	20	0.83	4	0.17	0	0.00	0.983	44	0.92	4	0.08	0.728*
	ctr with Hom*CFHR1* Δ (n=50)	44	0.88	6	0.12	0	0.00		94	0.94	6	0.06	
CFH c.1204T>C Tyr402His (rs1061170)	aHUS with anti-FH (n=29)	24	0.83	5	0.17	0	0.00	0.285	53	0.91	5	0.09	0.101*
	aHUS with anti-FH and Hom*CFHR1* Δ (n=23)	21	0.91	2	0.09	0	0.00	0.791	45	0.98	3	0.07	0.329*
	ctr with Hom*CFHR1* Δ (n=50)	48	0.96	2	0.04	0	0.00		98	0.98	2	0.02	
CFH c.2016A>G Gln572Gln (rs3753396)	aHUS with anti-FH (n=30)	25	0.83	4	0.13	1	0.03	0.055	54	0.9	6	0.10	*0.012**
	aHUS with anti-FH and Hom*CFHR1* Δ(n=24)	22	0.92	2	0.08	0	0.00	0.812	46	0.96	2	0.04	0.252*
	ctr with Hom*CFHR1* Δ (n=49)	48	0.98	1	0.02	0	0.00		97	0.99	1	0.01	
CFH c.2237-543G>A (rs1410996)	aHUS with anti-FH (n=28)	1	0.04	7	0.25	20	0.71	0.021	9	0.16	47	0.84	*0.009**
	aHUS with anti-FH and Hom*CFHR1* Δ (n=23)	0	0.00	3	0.13	20	0.87	0.867	3	0.07	43	0.93	0.384*
	ctr with Hom*CFHR1* Δ (n=49)	0	0.00	3	0.06	46	0.94		3	0.03	95	0.97	
CFH c.2808G>T Glu936Asp (rs1065489)	aHUS with anti-FH (n=30)	25	0.84	4	0.13	1	0.03	0.051	54	0.9	6	0.10	*0.016*
	aHUS with anti-FH and Hom*CFHR1* Δ (n=24)	22	0.92	2	0.08	0	0.00	0.507	46	0.96	2	0.04	0.246
	ctr with Hom*CFHR1* Δ (n=50)	49	0.98	1	0.02	0	0.00		99	0.99	1	0.01	
CD46 c.*783T>C (rs7144)	aHUS with anti-FH (n=30)	12	0.40	11	0.37	7	0.23	0.181	35	0.58	25	0.42	0.876
	aHUS with anti-FH and Hom*CFHR1* Δ (n=24)	10	0.42	10	0.42	4	0.17	0.544	30	0.63	18	0.38	1.000
	ctr with Hom*CFHR1* Δ (n=43)	16	0.37	23	0.53	4	0.09		55	0.64	31	0.36	

Table 4-6. Frequency of *CFH* and *CD46* susceptibility variants in aHUS patients with anti-FHs and super controls.

Prevalence of *CFH* rs3753394, rs800292, rs1061170, rs3753396, rs1410996, rs1065489, and *CD46* rs7144 in aHUS patients with anti-FHs and healthy controls with the homozygous *CFHR1* deletion (hom *CFHR1*Δ). The comparison was performed considering either the whole cohort of patients with anti-FHs (n=30) or aHUS patients with anti-FHs also carrying the homozygous *CFHR1* deletion (n=24). "M" is referred to the major allele, "m" is referred to the minor allele. *Fisher's exact test.

The association analysis was also performed considered the common polymorphisms in *C3* c.304C>G, p.Arg102Gly (rs1047286), *C3* c.941C>T, p.Pro314Leu (rs2230199), *CFB* c.94C>T, p.Arg32Trp (rs12614), *CFB* c.95G>A, p.Arg32Gln (rs641153) and *THBD* c.1418C>T, p.Ala473Val (rs1042579). No significant associations with the disease have been found with these polymorphisms despite a trend was present for *C3* c.304G 102Gly and c.941T 314Leu, which are in partial linkage disequilibrium (LD), caused by an increased number of heterozygous individuals for these variants (Table 4-7).

		MM	Freq MM	Mm	Freq Mm	mm	Freq mm	p-value	M	Freq M	m	Freq m	p-value
C3 c.304C>G Arg102Gly (rs2230199)	aHUS with anti-FH (n=27)	14	0.52	13	0.48	0	0.00	0.058	41	0.76	13	0.24	0.681
	aHUS with anti-FH and hom *CFHR1* Δ (n=22)	11	0.50	11	0.50	0	0.00	0.065	33	0.75	11	0.25	0.636
	ctr with hom *CFHR1* Δ (n=24)	17	0.71	5	0.21	2	0.08		39	0.81	9	0.19	
C3 c.941C>T Pro314Leu (rs1047286)	aHUS with anti-FH (n=27)	16	0.59	11	0.41	0	0.00	0.073	43	0.80	11	0.20	0.822
	aHUS with anti-FH and hom *CFHR1* Δ (n=22)	13	0.59	9	0.41	0	0.00	0.098	35	0.80	9	0.20	0.842
	ctr with hom *CFHR1* Δ (n=24)	18	0.75	4	0.17	2	0.08		40	0.83	8	0.17	
CFB c.94C>T Arg32Trp (rs12614)	aHUS with anti-FH (n=22)	17	0.77	4	0.18	1	0.05	0.565	38	0.86	6	0.14	1.000*
	aHUS with anti-FH and hom *CFHR1* Δ (n=19)	15	0.79	3	0.16	1	0.05	0.478	33	0.87	5	0.13	1.000*
	ctr with hom *CFHR1* Δ (n=20)	15	0.75	5	0.25	0	0.00		35	0.88	5	0.13	
CFB c.95G>A Arg32Gln (rs641153)	aHUS with anti-FH (n=22)	17	0.77	4	0.18	1	0.05	0.634	38	0.86	6	0.14	0.745*
	aHUS with anti-FH and hom *CFHR1* Δ (n=18)	15	0.83	3	0.17	0	0.00	0.997	33	0.92	3	0.08	1.000*
	ctr with hom *CFHR1* Δ (n=19)	15	0.79	4	0.21	0	0.00		34	0.89	4	0.11	
THBD c.1418C>T Ala473Val (rs1042579)	aHUS with anti-FH (n=27)	16	0.59	11	0.41	0	0.00	0.850	43	0.80	11	0.20	0.614
	aHUS with anti-FH and hom *CFHR1* Δ (n=22)	14	0.64	8	0.36	0	0.00	0.979	36	0.82	8	0.18	0.854
	ctr with hom *CFHR1* Δ (n=24)	17	0.71	7	0.29	0	0.00		41	0.85	7	0.15	

Table 4-7. Frequency of *C3, CFB,* and *THBD* susceptibility variants in aHUS patients with anti-FHs and super controls.

Prevalence of *C3* rs1047286 and rs2230199, *CFB* rs12614 and rs64115, and *THBD* rs1042579 in aHUS patients with anti-FHs and healthy controls with the homozygous *CFHR1* deletion (hom *CFHR1*Δ). The comparison was performed considering either the whole cohort of patients with anti-FHs (n=30) or aHUS patients with anti-FHs also carrying the homozygous *CFHR1* deletion (n=24). "M" is referred to the major allele, "m" is referred to the minor allele. *Fisher's exact test.

4.3.10 CFH haplotypes

Since strong LD is present in the *CFH* region (Figure 4-4), *CFH* haplotypes have been studied in the 24 aHUS patients with anti-FHs and homozygous deletion of *CFHR1* and the 50 super controls. The expectation maximization algorithm by Haploview software was used to estimate each *CFH* haplotype frequency. The minimal informative SNPs within *CFH* gene considered for this analysis were rs3753394 (promoter), rs800292 (exon 2), rs1061170 (exon 9), rs3753396 (exon 14), rs1410996 (intron 15), and rs1065489 (exon 19), as described in Rodriguez-De Cordoba *et al.*.[88]

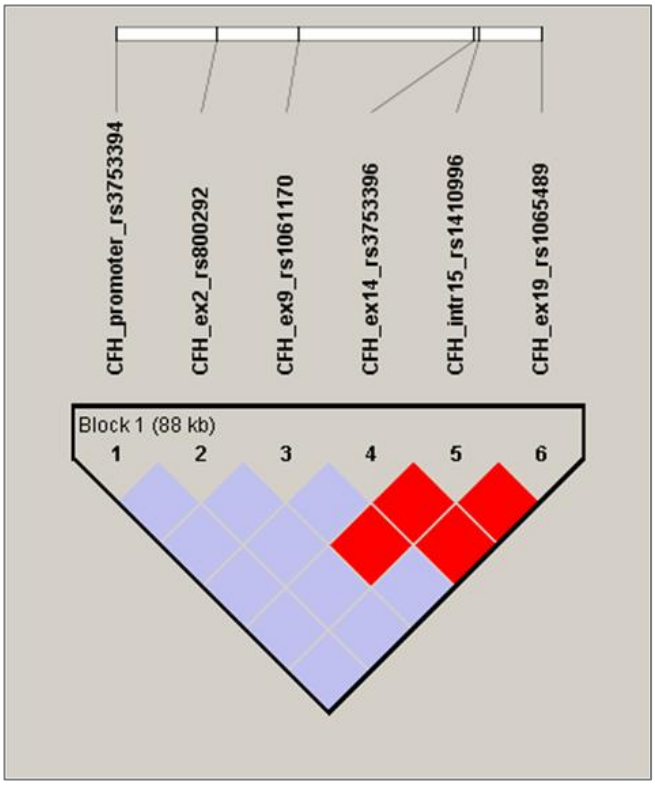

Figure 4-4. LD in *CFH* locus.

The analysis was obtained with Haplotype software using 6 *CFH* SNPs as markers (rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs1065489). The red boxes meant complete LD with LOD ≥2 and blue boxes a LOD<2 and D' =1.

The most frequent *CFH* haplotypes were the CGTAAG (H4a *CFH* haplotype), which was estimated to have an allele frequency of 42.7% in aHUS patients and of 54.7% in super controls and the TGTAAG (H4b *CFH* haplotype) which was estimated to have an allele frequency of 40.6% in aHUS patients and of 36.3% in super controls (Table 4-8). H1 *CFH* haplotype (CGCAGG) that is reported in literature to be the most common *CFH* haplotype in healthy subjects was estimated to have a frequency of 4.2% in aHUS patients and 2% in super controls (Table 4-8). The H3 *CFH* haplotype, (TGTGGT), that is described strongly associated with aHUS, was estimated in 4.2% of aHUS patient chromosomes and in 1% of super control chromosomes (Table 4-8). The H2 *CFH* haplotype (CATAAG) were estimated to be more frequent in aHUS patients compared to super controls (haplotype frequency = 7.3% and 5.3%, respectively, Table 4-8). In summary, no significant association has been found between *CFH* haplotypes and anti-FH-mediated aHUS when I considered as reference sample the "super controls".

Estimated *CFH* haplotype	Haplotype reference number	Haplotype frequency		p-value
		aHUS patients with anti-FH	Super controls	
CGTAAG	H4a	0.427	0.547	0.174
TGTAAG	H4b	0.406	0.363	0.616
CATAAG	H2	0.073	0.053	0.628
CGCAGG	H1	0.042	0.02	0.447
TGTGGT	H3	0.042	0.01	0.201

Table 4-8. Identification of *CFH* haplotypes.

CFH haplotypes were identified through Haploview software in 24 aHUS patients with anti-FHs and 50 healthy controls both carrying the homozygous deletion of *CFHR1*. The estimated frequencies of each *CFH* haplotype in aHUS patients and super controls were also shown. The minimal informative SNPs within *CFH* gene considered for this analysis were rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs1065489.

Interestingly, in patients with anti-FHs, H4a and H4b *CFH* haplotypes were found in strong LD with the *CFHR3-CFHR1* deletion (Table 4-9). The *CFH* H2 was also found to be in association with the *CFHR3-CFHR1* deletion but the allele frequency was lower than *CFH* H4a and H4b haplotypes (Table 4-9). Instead, the *CFHR1-CFHR4* deletion in our cohort of patients was associated with *CFH* H5 and H3 haplotypes (Table 4-9).

Estimated *CFH* haplotype	Haplotype reference number	Δ*CFHR3-CFHR1*	Δ*CFHR1-CFHR4*	Frequency mean
CGTAAG	H4a	d	D	0.428
TGTAAG	H4b	d	D	0.321
CATAAG	H2	d	D	0.090
TGTGGT	H3	D	D	0.068
TGCAGG	H5	D	d	0.036
CGCAGG	H1	D	D	0.023
TGTGGT	H3	D	d	0.018

Table 4-9. LD between *CFH* haplotypes and *CFHR3-CFHR1* or *CFHR1-CFHR4* deletions in aHUS patients with anti-FHs.

(D = wild type allele, d = allele with deletion).

4.3.11 Analysis of the genetic variants in *CFH* promoter that could predispose to the loss of tolerance toward FH

In an attempt to investigate possible genetic factors predisposing to lack of tolerance toward FH/FHR1 and anti-FHs, I studied the sequence of the *CFH* distal promoter in aHUS patients with anti-FHs looking for possible variants in AIRE "autoimmune regulator" consensus regions. AIRE, is the most important DNA binding molecule expressed in the thymus involved in immune regulation and tolerance. The analysis through Matinspector software (Genomatix) revealed the presence of two sequences predicted with a good score as AIRE consensus motifs (Figure 4-5). Both sequences were compatible with possible AIRE binding sequences as described in Kumar *et al.*[137] I sequenced a region around 600 bp upstream the *CFH* proximal promoter including the two predicted AIRE consensus regions but I did not find any mutation or common variant in aHUS

patients with anti-FHs, with the only exception of a common variant c.1-778 A>G in an Argentinean patient (rs35928059, MAF = 32% in South America population). No variations have been observed in two DNA sequences predicted as AIRE consensus regions.

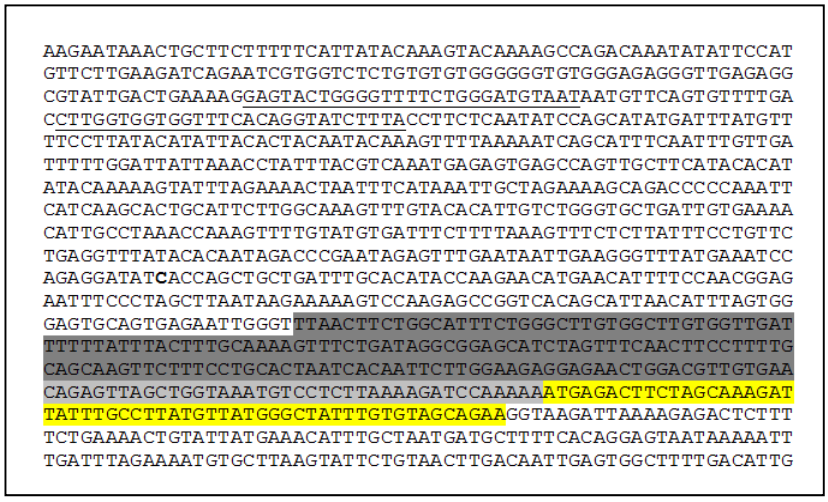

Figure 4-5. The sequence of *CFH* promoter.

Exon 1 (yellow), basal promoter (light grey), proximal promoter (dark grey) and two AIRE consensus sequences predicted by Matinspector software - Genomatix (underlined) were shown.

4.4 Discussion

In this study I found 30 out of 305 unrelated aHUS patients with anti-FHs. The prevalence (9.8%) was concordant with previously published data (4.6-13.3%).[43, 98, 109] I have also found 1 out of 111 controls with anti-FHs (0.09%) as compared to 1-4% healthy positive subjects reported in literature.[109] In our healthy controls the lower prevalence of anti-FHs is likely determined by the systematic approaches adopted to eliminate the false positive samples and to increase the ELISA specificity.

CFHR1 deficiency is present in 83.3% of aHUS patients with anti-FHs. This deficiency is caused by the presence of the homozygous *CFHR3-CFHR1* deletion in 66.7% of patients, the compound heterozygous *CFHR3-CFHR1*/*CFHR1-CFHR4* deletions in 13.3% of patients, and the heterozygous *CFHR3-CFHR1* deletion combined with a stop codon in *CFHR1* gene (c.104delAfsX, p.D35fsX36) that leads to a complete FHR1 deficiency in another patient. Several studies reported that 79-89% of aHUS patients with anti-FHs were carriers of deletion or nonsense mutations in *CFHR1*.[43, 109] All these data indicate that in aHUS patients the *CFHR1* absence strongly predisposes to anti-FHs development and to aHUS.

Of note, FHR1 deficiency is not an absolute prerequisite for development of anti-FHs. Similar to data showed in Moore and colleagues, who reported 3 out of 13 patients without copy number defects in *CFHR3* and *CFHR1,* I have found 3 patients with anti-FHs carrying one copy of *CFHR1* and 2 patients with 2 copies of *CFHR1*. Altogether these results suggest that in a few aHUS patients the development of anti-FHs is not dependent on FHR1 deficiency.[107]

In this cohort of patients, most of anti-FHs predominantly targeted the C terminal of FH, a crucial domain involved in the regulation of AP complement activation at the cell surface, and this specificity is not strongly correlated with the presence of homozygous deletion of *CFHR1*. At variance, Nozal *et al.* reported that FH autoantibodies from 13 aHUS patients with FHR1 deficiency mainly recognized SCR19-20 but in 6 patients with at least one copy of *CFHR1* the anti-FH specificity was more heterogeneous.[176] It was well demonstrated that FH C terminus is a main domain for regulatory activity. *CFH* mutations in aHUS patients cluster in SCR 19-20 and a transgenic murine model with deficiency of FH SCR16-20 spontaneously manifests aHUS.[93, 177] Nozal *et al.* have demonstrated that FH 1183-1189 and 1210-1215 within SCR19-20 are the main antibody binding sites.[176] Bhattacharjee *et al.* also described autoantibody epitopes in SCR20, next to Ser1191 and Val1197 aminoacids, where FH binds heparin, endothelial cells and common microbes.[48, 121]

In this study I have evidenced that among about 1400 Caucasian healthy controls, mostly Italian, 5% are carriers of the homozygous *CFHR1* deletion. Only 1 out of 13 healthy controls with no copies of the *CFHR1* gene, resulted positive (0.08%), while among 98 controls with at least one copy of *CFHR1* none showed anti-FHs. This finding suggests that also in healthy subjects the *CFHR1* deletion could predispose to the anti-FH development but this condition is not sufficient for the aHUS manifestation. This hypothesis is supported by the published evidence of few unaffected siblings reported with the homozygous *CFHR1* deletion showing elevated anti-FH titers.[110]

Since the homozygous *CFHR1* deletion is present in the 5% of healthy population, it cannot be considered sufficient alone to determine the anti-FH – mediated aHUS which has a prevalence of 1:1,000,000. This observation suggests that the aHUS caused by anti-FHs is a complex disease in which multiple hits (such as mutations, common variants, and triggers) are necessary for the disease to manifest.

Indeed, in this study, 11 patients with anti-FHs were also carriers of mutations in complement genes (37%). This prevalence was higher compared to that present in literature (14.7%) which is referred to 95 published cases. *CFH*, *CD46*, and *THBD* mutations are the more abundant (both presented 10% of prevalence in our patients). Interestingly, the *CFH* c.2850G>T p.Gln950His, that I have identified in 1 patient, was also found in other 4 patients without anti-FHs of our aHUS cohort (carrier frequency = 0.009). Of note, all patients with *CFH* c.2850T variant showed the *CFHR1* deletion at least in one chromosome suggesting the presence of a strong LD between this mutation and the deletion of *CFHR1*. However, only one of the 4 patients who carried this variant had anti-FHs. This observation could lead to consider the *CFH* c.2850T like a risk variant for the atypical HUS but not a predisposing factor for the autoimmunity against factor H. This conclusion was supported by evidences obtained by Mohlin and colleagues who showed that serum of patients carrying the *CFH* c.2850T caused moderate but statistically significant lysis of the erythrocytes during the hemolytic assay.[168] In the literature, concomitance mutations are reported in about 25% of aHUS patients with *CFI* mutations but here no patients showed defects in *CFI* gene.[86] Interestingly, anti-FHs were also found in combination with *C3* and *THBD* mutations. The presence

of mutations in complement genes in aHUS patients with anti-FHs have also been reported in other studies and together with my findings document that the combination of both acquired and genetic factors in some patients could be crucial for the aHUS development.[107]

CFH common variants were described as additional genetic risk factors that can increase the aHUS penetrance.[86, 87] Here, an association analysis has been performed using "super controls", healthy individuals with the homozygous *CFHR1* deletion, a risk factor for aHUS associated to anti-FHs, but no significant associations were observed. The lack of *CFH* polymorphisms associated to an increased aHUS risk, such as *CFH* c.1-332C>T (rs3753394), c.2016A>G Gln572Gln (rs3753396), and c.2808G>T Glu936Asp (rs106548), was caused from the absence of the predisposing *CFH* H3 haplotype in these patients with anti-FHs. In this study, the *CFH* H4a (CGTAAG) and *CFH* H4b (TGTAAG) haplotypes were found in most patients with anti-FHs but also in "super controls". Indeed, the *CFHR3-CFHR1* deletion was in LD with both *CFH* H4 haplotypes (CGTAAG and TGTAAG). The other *CFH* haplotype that tags the *CFHR3-CFHR1* deletion in our patients is the H2 haplotype (CATAAG) which shares with *CFH* H4 haplotype the last three variants (AAG), respectively rs3753396 (*CFH* exon 14), rs1410996 (*CFH* intron 15), rs1065489 (*CFH* exon 19). Spencer *et al.* observed that the rs3753396 "A" variant and the rs1065489 "G" variant were in LD with the *CFHR3-CFHR1* deletion.[178] All these data evidence that a strong LD exists between the last portion of *CFH* sequence and the *CFHR3-CFHR1* deletion. A limited number of DNA breakpoints in *CFH* genomic region are probably present in aHUS patients and controls with *CFHR3-CFHR1* deletion

who likely share the same *CFH* sequence in this chromosome region. These data also suggest that in aHUS patients with anti-FHs there should not be peculiar variants at least in the second portion of *CFH* sequence compared to healthy controls which could have a role in the development of anti-FHs. Similarly, the FH H3 haplotype, that has been previously reported associated with aHUS, (29% in aHUS patients *vs* 20.7% in healthy controls, p-value = 0.013 in Rodriguez-De Cordoba *et al.*[88]), was not overrepresented in our aHUS patients with anti-FHs and it showed a similar allele frequency in patient and super control groups (2.1% *versus* 1%).[88] Thus, factors predisposing to loss of tolerance toward FH have probably to be looked for in other genomic regions.

Also the *CD46* GGAAC haplotype has been reported to be associated with an increased risk of aHUS. Moore *et al.* found that 10 out of 13 aHUS patients with anti-FH (77%) carried the *CD46* GGAAC haplotype and suggested that it could be a possible susceptibility factor for the development of aHUS in patients with anti-FHs.[107] Here, the *CD46* C variant of the rs7144 has been used as a marker for the at-risk *CD46* haplotype [94] but no significant difference in the prevalence of the C variant was found between patients with the factor H autoantibodies and the "super controls" reference group.

The C3 102Gly variant was overrepresented in aHUS patients with anti-FHs compared to control group caused by the presence of more than two-fold heterozygous in cases than healthy subjects, but the result is not statistically significant. Of note, The C3 102Gly was associated with AMD, IgA nephropathy, systemic vasculitis, kidney graft dysfunction, and DDD.[165, 167, 178, 179] This association was not described in aHUS and was not confirmed in another

published study on DDD or other membranoproliferative glomerulonephritis.[55] Of note, the C3 102G variant has a functional effect because it is demonstrated to activated AP more efficiently in hemolytic assay and in its presence the FH cofactor activity was reduced favoring AP activation.[92]

In order to identify the genetic factors that can determine the loss of tolerance and autoimmunity against FH in subjects with the homozygous *CFHR1* deletion, I focused on the *CFH* promoter looking for possible variants that can influence the genetic expression in thymus, the crucial organ for immunological tolerance.[138, 139] First of all, I studied the *CFH* SNP rs3753394, which is located in an NF-kB responsive element within the *CFH* promoter and it was previously reported to be associated with aHUS.[87, 180] However, as I have described above, I found that rs3753394 T variant was not associated with aHUS caused by anti-FHs using as reference group healthy controls with the homozygous *CFHR1* deletion. *In silico* analyses of *CFH* promoter region showed the presence of two sequences predicted as possible consensus for the binding of AIRE, an autoimmune regulator that is expressed by mTEC cells in thymus and is crucial for the expression of the tissue specific antigens and the maintenance of the central tolerance.[181] The sequencing of the *CFH* distal promoter region, including the two predicted AIRE binding sequences, did not reveal any mutations in patients with anti-FHs suggesting that this region is not involved in the loss of the tolerance for FH antigene.

In conclusion in this chapter of the thesis, I have documented that the anti-FH mediated aHUS is a complex disease, in which the FHR1 deficiency represents a strong risk factor for the anti-FH development and in which additional genetic

defects are required for the disease to manifest. However, the presence of the FHR1 deficiency is not enough for the anti-FH development. This consideration is supported by evidences according to which autoimmune diseases are often complex disorders caused by polygenic factors each conferring a small percentage of risk that leads to a susceptible state.[182, 183] Infections or other trigger events could perturb this status promoting the loss of tolerance and the development of autoimmunity.

5 Clinical features of patients with anti-FH antibodies

5.1 Introduction

Atypical Hemolytic Uremic Syndrome (aHUS) is associated to the presence of anti-FHs in about 5-14% of patients in European cohorts.[98, 106, 107, 110] Anti-FHs mainly develop in the context of FHR1 deficiency and caused the impairment of factor H functions compromising the self surface protection from complement activation and the development of the disease.[115]

Only few clinical reports with the description of aHUS patients with anti-FHs are available in the literature. The large majority of cases have been evaluated during the acute phase of the disease before any treatment but in patients analyzed at follow-up anti-FHs remained detectable also during the convalescence.[109] Anti-FHs affect mainly children between 5 and 15 years and the age at the disease onset is different from that described in children affected by aHUS carrying mutations in *CFH* or *DGKE* mutations, which is in the first years of life.[106, 109] Most of aHUS cases with anti-FHs present prodromal signs, especially upper respiratory tract infections or gastrointestinal signs (such as abdominal pain, vomiting, and diarrhea).[109] Low C3 levels are observed in 41-67% of patients indicating the AP of complement is activated.[109] On the contrary, C4 is normal in the large majority of them.[109] The FH antigen levels are not reported to be correlated with the anti-FH titer although in the acute phase of the disease they

decrease in few patients because FH is bound to autoantibodies forming immune-complexes.[109, 114]

Relapses occur in a large proportion of patients with anti-FH associated aHUS.[109] Moreover, about 30% of patients develop ESRD.[106, 110] Plasma exchange is more effective than plasma infusion to induce hematological remission, although 35% of patients treated with plasma exchange developed renal failure.[109] On the contrary, the combination of plasma exchange and immunosuppression is reported to reduce the risk of aHUS relapses and to protect the renal function.[109] Indeed, only 14.3% of patients treated with plasma exchange in combination with immunosuppression manifested ESRD.[109] Eculizumab has been successfully adopted in few cases leading to the remission of the disease, discontinuation of plasma treatment, and renal recovery in the large majority of cases.[109] Finally, plasma exchange and immunosuppression are effective administered before the transplant to prevent the recurrence of the disease on the graft in most patients. Eculizumab is also used to prevent aHUS recurrence after renal transplantation but only a limited number of cases are available in the literature.

In this chapter I described the clinical and biological features of 30 patients affected by aHUS and positive for anti-FHs, of whom 25 were also carriers of the FHR1 deficiency.

5.2 Specific aim

To describe the clinical features of 30 patients with anti-FH associated aHUS.

5.3 Results

Clinical data concerning the acute (n=4) or the remission phase (n=26) were obtained for all the 30 aHUS patients with anti-FHs (Table 5-1).

The mean anti-FH titers was 1164.76±1189.51 AU/mL for samples collected during the acute phase of the disease (n=4) and 521.83±504.34 AU/mL for samples collected during the remission phase (n=26; ANOVA, p-value = 0.062). C3 and C4 level data were available for 28 patients (Table 5-1). Fourteen patients (46.7%) showed lower than normal C3 levels and in most of them C3 was measured during the convalescence (mean ± SD, 90 mg/dL ± 34, normal range 90-180 mg/dL). On the contrary, C4 levels were normal in all patients (mean ± SD, 28 mg/dL ± 13, normal range 10-40 mg/dL). FH have been measured in 24 patient samples and resulted in normal range in all cases (mean ± SD, 344 mg/L ± 110, normal range 172-507 mg/L, Table 5-1) with the only exception of patient number 14 (studied in remission and with a high antibody titer, 1557.3 AU/mL) who showed 80 mg/L FH. The mean of FH levels did not differ between samples collected during the acute and the remission phase of the disease (340±49 mg/L n=3 and 345±117 mg/L, n=21, respectively; ANOVA, p-value = 0.951) and the FH levels were not correlated to the antibody titer ($r^2 = 0.0085$).

ID	Gender	Ethnicity	Age at disease onset (years)	Phase	Disease triggers	Anti-FH titer (AU/mL)	C3 level (90-180 mg/mL)	C4 level (10-40 mg/mL)	FH level (172-507 mg/L)
1	F	Caucasian (Eu)	7.0	Rem	Vomiting	453.4	30	12	na
2	M	Caucasian (Eu)	7.7	Rem	Flu like	381.6	141	27	na
3	F	Caucasian (Eu)	7.6	Rem	Upper respiratory tract infection	280.8	112	29	na
4	F	Caucasian (Eu)	8.0	Rem	None	513.2	na	na	na
5	M	Caucasian (Eu)	8.8	Rem	Vomiting	146.6	85	25	349
6	M	Caucasian (Eu)	6.2	Rem	Flu like	243.0	81	26	309
7	M	Caucasian (Eu)	5.7	Rem	None	572.0	94	17	312
8	M	Caucasian (USA)	6.7	Rem	na	1646.1	77	31	283
9	F	Caucasian (Eu)	7.6	Rem	Vomiting	876.7	70	19	302
10	F	Caucasian (Eu)	6.6	Acute	Upper respiratory tract infection	1314.9	103	25	397
11	F	Caucasian (Eu)	5.4	Acute	Vomiting	2781.0	96	28	na
12	M	Caucasian (Eu)	7.8	Rem	Vomiting	342.9	185	62	307
13	F	Caucasian (Eu)	12.8	Rem	None	1590.4	118	22	686
14	F	Caucasian (Eu)	15.5	Rem	Vomiting and fever	1557.3	35	27	80
15	M	Caucasian (Eu)	11.0	Rem	Vomiting	862.3	100	30	438
16	F	Caucasian (USA)	10.5	Rem	Vomiting and fever	877.4	na	na	308
17	F	Caucasian (USA)	5.3	Rem	Flu like	189.15	106	70	446
18	M	Caucasian (Eu)	10.1	Rem	Vomiting	531.2	75	14	463
19	F	Caucasian (Eu)	8.8	Rem	na	226.6	78	20	374
20	F	Caucasian (Eu)	30.2	Rem	Caesarean	111.8	149	38	432
21	F	Hispanic	17.6	Rem	Oral contraceptive	96.6	39	25	244
22	M	Caucasian (Eu)	7.6	Rem	Upper respiratory tract infection	102.1	89	39	362
23	M	Asian	9.2	Acute	Vomiting and fever	123.1	54	29	307
24	M	Jewish	5	Rem	None	77.6	126	35	226
25	M	Persian	15.0	Acute	Upper respiratory tract infection	440.15	95	27	317
26	M	Hispanic	6.5	Rem	None	141.2	96	27	362
27	M	Caucasian (Eu)	7.1	Rem	Upper respiratory tract infection	65.2	65	14	246
28	F	Caucasian (Eu)	8.1	Rem	Vomiting and fever	57.1	99	22	372
29	M	African-Arab	1.3	Rem	Upper respiratory tract infection	303.9	68	11	na
30	M	Caucasian (Eu)	6.9	Rem	Vomiting	1321.7	63	31	337

Table 5-1. Clinical and biological data of 30 patients with anti-FH associated aHUS.

F: female; M: male; Phase: indicates the phase of the disease when the samples were collected, remission phase (Rem) or acute phase (Acute). na = not available

The large majority of patients with anti-FHs of our cohort were Caucasians.They showed an equal gender ratio, with 53.3% males (Table 5-1). The median age of disease onset was 7.7 [6.6-9.9] years. All patients with anti-FHs but one

developed aHUS during childhood or adolescence (96.7%). A woman developed anti-FH aHUS at 30 years of age after the caesarean delivery and a child manifested aHUS at 16 months (he also carries a *THBD* mutation, see Table 4-4). The distribution of the age at disease onset in patients with anti-FHs was unimodal with a clear incidence peak around 8 years of age (Figure 5-1, panel A). The distribution of age at the disease onset was different from that of aHUS patients negative for anti-FHs and carrying mutations in genes such as *CFH*, *MCP*, *CFI*, *THBD*, *C3*, *CFB* and *DGKE*, who showed a bimodal distribution with a peak of earlier onset of aHUS, often before 4 years of age, and a second peak during adulthood (Figure 5-1, panel B).

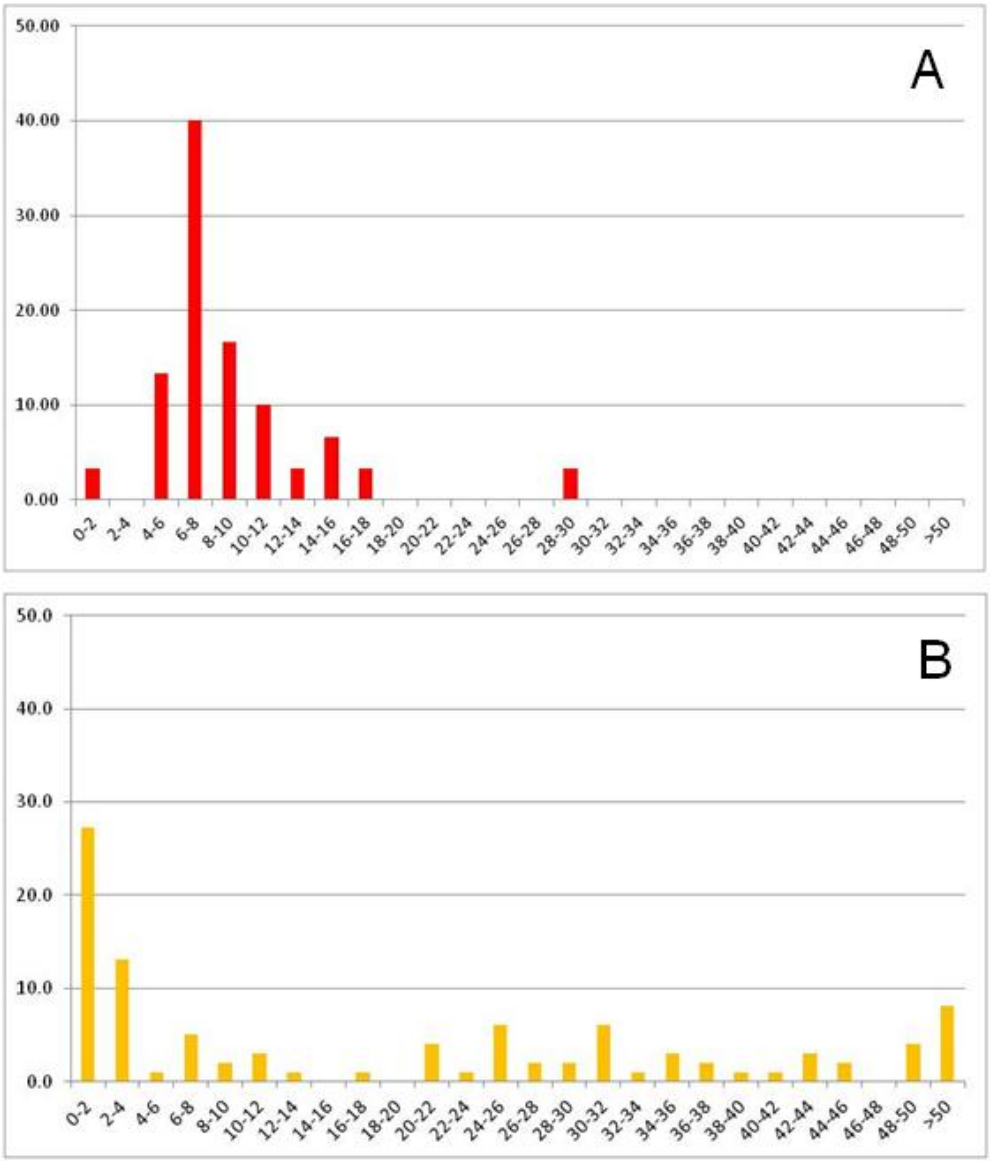

Figure 5-1. Distribution of age at disease onset in aHUS patients analyzed for anti-FHs.

In panel A patients positive for anti-FHs (n=30) and in panel B patients negative for anti-FHs and with mutations in complement genes (n=99).

Since in the chapter 4 of this thesis, I have shown that the homozygous *CFHR1* deletion was also more abundant in aHUS patients without anti-FHs compared to healthy controls (see Table 4-2), I made further analyses to better understand the significance of such association. The percentage of samples analyzed for anti-FHs during the acute phase of the disease was 13.3% (4 out of 30) in the group of

patients positive for anti-FHs and 19% (5 out of 26) in the group of patients negative for anti-FHs and carrying the deletion of *CFHR1*. Thus, most samples were collected during the remission phase in both groups indicating that in patients with anti-FHs the antibodies remain detectable after the acute phase, and suggesting that most of the anti-FH negative samples were likely true negatives. Moreover, in the group of patients negative for anti-FHs and carrying the homozygous *CFHR1* deletion, the median age at the disease onset was significantly different from that of patients positive for anti-FHs carrying the homozygous deletion of *CFHR1* (median = 24.1 [7.6-38.6] *versus* 7.7 [6.5-9.1] years, Mann-Whitney test, p-value = 0.017). The distribution of age at the disease onset also appeared different between these groups of patients (Figure 5-2).

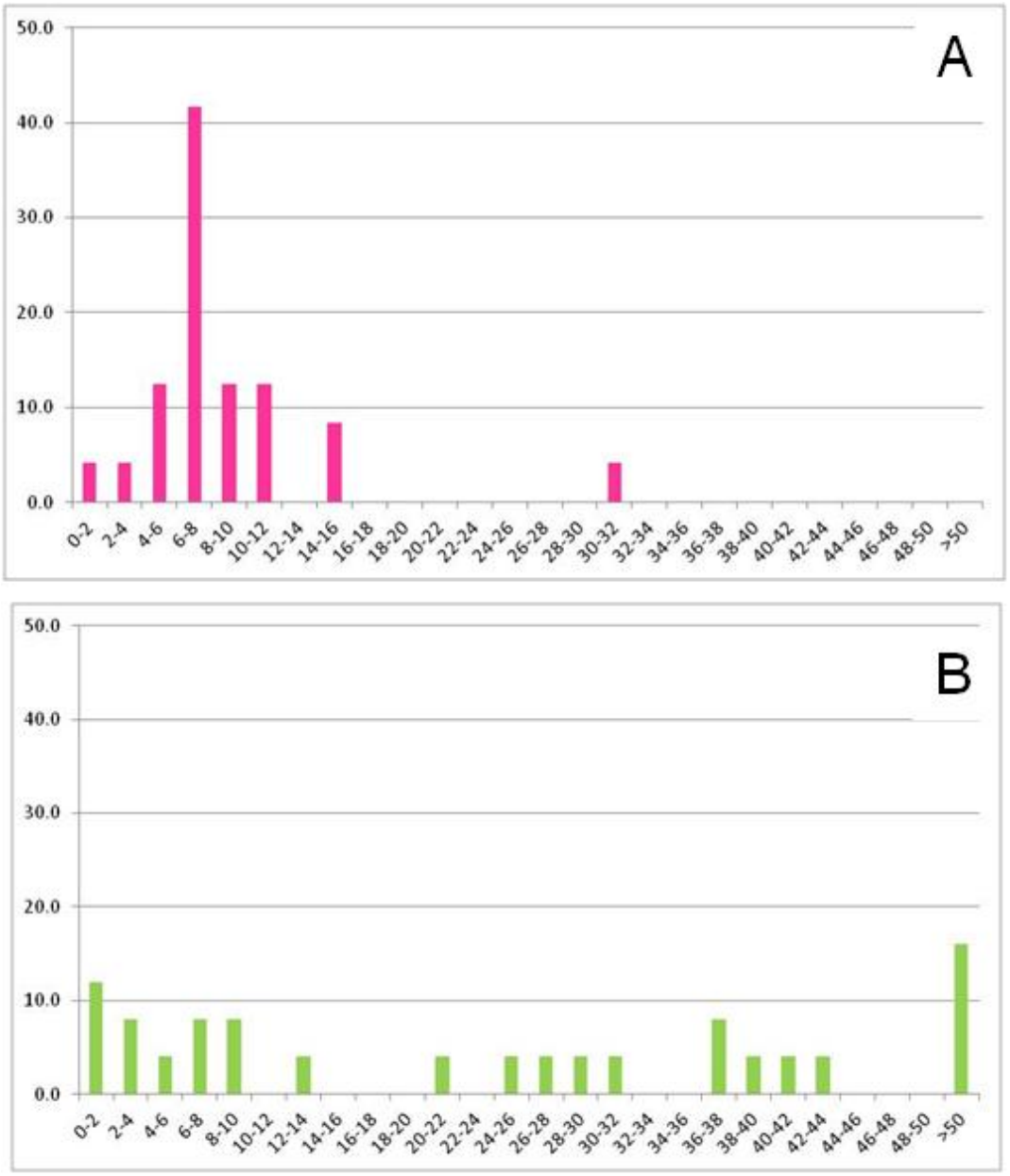

Figure 5-2. Distribution of age at disease onset in aHUS patients carrying the homozygous deletion of *CFHR1*.

In panel A patients positive for anti-FHs (n=24) and in panel B patients negative for anti-FHs (n=26).

On the contrary, no difference was found in the age of disease onset between patients negative for anti-FHs and carrying the homozygous *CFHR1* deletion and

patients with mutations in complement genes (median = 24.1 [7.6-38.6] *versus* 10.6[1.32-31.9] years, Mann-Whitney test, p-value = 0.094, Figure 5-3).

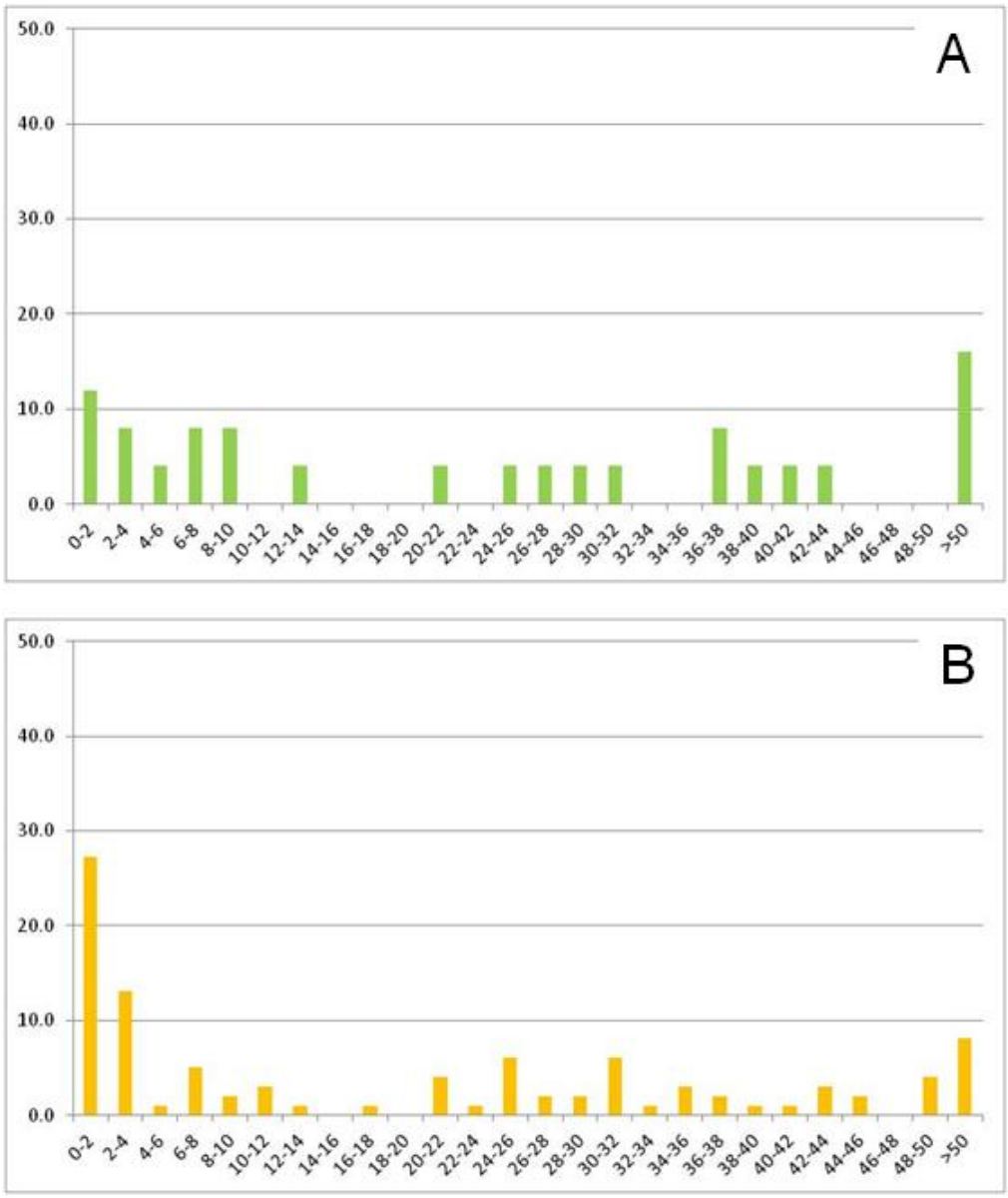

Figure 5-3. Distribution of age at disease onset in aHUS patients negative for anti-FHs**.**

In panel A patients with the homozygous deletion of *CFHR1* (n=26) and in panel B patients with mutations in complement genes (n=99).

By logistic regression I have estimated the risk for an aHUS patient to present anti-FHs when the homozygous deletion of *CFHR1* and the age at disease onset between 4 and 12 years were present. Twenty-four out of 30 aHUS patients with anti-FHs (80%) *versus* 26 out of 275 aHUS patients without anti-FHs (9.4%) showed homozygous deletion of *CFHR1* (OR [95% CI] = 38.31 [14.354-102.231], p-value = 4.88×10^{-22}, Table 5-2). Twenty-four out of 30 aHUS patients with anti-FHs (80%) *versus* 27 out of 275 aHUS patients without anti-FHs (9.8%) developed the disease between 4 and 12 years of age (OR [95% CI] = 36.74 [13.805-97.87], p-value = 1.67×10^{-21}, Table 5-2). Moreover, the presence of both the homozygous *CFHR1* deletion and the age at disease onset between 4 and 12 years further increased the risk to have anti-FHs (OR [95% CI] = 108 [33.665-346.472], p-value = 6.94×10^{-33}, Table 5-2).

	anti-FH Ab					
	Positive	**Negative**	**Sensitivity**	**Specificit**	**OR [CI 95%]**	**p-value**
Hom *CFHR1* Δ	24	26	0.80	0.91	38.31 [14.354-102.231]	4.88×10^{-22}
No hom *CFHR1* Δ	6	249				
Onset 4-12 years	24	27	0.80	0.90	36.74 [13.805-97.87]	1.67×10^{-21}
Onset <4 or ≥13 years	6	248				
Hom *CFHR1* Δ and onset at 4-12 years	20	5	0.67	0.98	108 [33.665-346.472]	6.94×10^{-33}
Others	10	270				

Table 5-2. Estimation of risk for an aHUS patient to have anti-FHs in the presence of homozygous deletion of *CFHR1* and in presence of the age at disease onset between 4 and 12 years.

OR = odds ratio

Prodromal signs were observed in the majority of the anti-FH positive patients. Six patients (21%) had upper respiratory tract infections (1 of them with vomiting and 2 with fever), 12 patients (43%) showed gastrointestinal symptoms (4 of them with fever), and 3 flu-like symptoms. One patient developed post-partum aHUS and another one during oral contraception. Five patients did not show any trigger event and for other 2 patients trigger data were not available (Table 5-1).

Fourteen patients with anti-FHs developed relapses of the disease (47%) and there was no difference between carriers or non-carriers of abnormalities in complement genes (45% vs 42%, respectively; Fisher's exact test, p-value = 1.000, Table 5-3). Overall, 14 patients (46.7%) developed end stage renal disease (ERSD), of whom 6 carrying genetic defects (Table 5-3).

	Outcome			Treatment					
ID	Relapses	ESRD	Death	Plasma infusion	Plasma exchange	Dialysis	Steroids	Kidney trasplant	Recurrence on graft
1	No	No	No	Yes	Yes	Yes	Yes	No	
2	No	No	No	Yes	No	No	Yes	No	
3	Yes	No	No	No	Yes	Yes	No	No	
4	No	No	No	Yes	Yes	No	No	No	
5	No	Yes	No	No	No	Yes	No	Yes	No
6	Yes	Yes	No	No	Yes	Yes	No	No	
7	No	No	No	No	Yes	No	No	No	
8	No	Yes	No	No	Yes	Yes	No	No	
9	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No
10	Yes	No	No	Yes	No	No	No	No	
11	Yes	No	No	Yes	No	No	No	No	
12	Yes	Yes	No	No	Yes	No	Yes	No	
13	Yes	No	No	Yes	Yes	No	No	No	
14	No	Yes	No	Yes	No	Yes	No	No	
15	No	Yes	No	Yes	Yes	Yes	Yes	No	
16	No	No	No	No	Yes	Yes	No	No	
17	Yes	Yes	No	No	No	Yes	No	No	
18	Yes	No	No	Yes	Yes	No	Yes	No	
19	No	Yes	No	No	No	Yes	No	No	
20	No	No	No	Yes	No	No	No	No	
21	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes
22	Yes	Yes	No	No	Yes	Yes	Yes	No	
23	Yes	Yes	No	Yes	Yes	Yes	Yes	No	
24	Yes	Yes	No	No	Yes	Yes	Yes	No	
25	Yes	No	No	na	na	na	Yes	No	
26	No	No	No	Yes	No	No	No	No	
27	No	No	No	No	No	Yes	No	No	
28	No	No	No	No	No	Yes	No	No	
29	No	No	No	Yes	No	No	No	No	
30	Yes	Yes	No	Yes	Yes	Yes	No	No	

Table 5-3. Outcome and treatment of 30 patients with anti-FH associated aHUS.

ESRD is End Stage Renal Disease. na = not available

Twenty-three out of 30 patients were treated with either fresh frozen plasma infusion or with plasma exchange (Table 5-3). Ten out of 23 patients treated with plasma developed aHUS relapses (43.5%), and 10 (43.5%) developed end stage renal disease (ERSD). Eleven patients were treated with steroids (of whom 9 in combination with plasma therapy) and 7 developed ESRD (of whom 6 have received steroids and plasma combined therapies) and 4 underwent full remission (Table 5-3). Three patients received kidney transplantation and disease recurrence

was observed in one of them. Of note, this patient was also a carrier of a C3 heterozygous mutation, p.Arg592Trp (Table 5-3, also see Table 4-4).

5.4 Discussion

In this chapter I have reported the clinical and biological data of 30 patients with aHUS and anti-FHs. All these patients were recruited in the last two decades through the International Registry of HUS/TTP of our clinical centre in Ranica and belong to a multicentre study. For all of them the diagnosis of aHUS caused by anti-FHs was made retrospectively and the large majority of patient data was referred to the convalescence phase. Therefore, it is important to note that some data limitations are present caused by the retrospective nature of this study.

In our cohort of patients, the presence of anti-FHs leads to C3 consumption, as usually described in aHUS patients carrying mutations and suggesting an activation of the AP of complement. Nevertheless, the C3 levels are only slightly reduced indicating that a trend to C3 normalization occurred during the remission phase of the disease. Despite the presence of anti-FHs in the patient plasma/serum samples, the levels of FH are normal, probably because the large majority of samples are collected during the convalescence when the autoantibody titer is decreased.

In our cohort, anti-FHs are observed especially in children with a peak of disease onset around 8 years, which is different from the age of aHUS onset which occurs in patients carrying mutations in complement genes.[62, 100] This finding is consistent with data observed in other published cohorts of patients in whom the anti-FH aHUS is prevalent among cases with onset between 5 and 15 years.[106, 109, 184]

In our aHUS cases positive for anti-FHs, the age of aHUS onset is different from that of patients negative for anti-FHs but carrying the homozygous *CFHR1* deletion, which is more similar to the age of onset in patients carrying mutations. Thus, I would hypothesize that in this group of patients the disease is caused by genetic defects.

In this study, 60% of aHUS patients with anti-FHs present prodromal signs, mostly upper respiratory tract infections and gastrointestinal symptoms. Published clinical reports show that respiratory infections, abdominal pain and vomiting are the most common triggers in aHUS patients with anti-FHs.[106, 109] These data can support the hypothesis according to which the autoimmunity toward FH could develop as a result of an infection in subjects with a predisposed background (the absence of FHR1).

The percentage of relapses (47%) in our cohort is similar to that reported in other studies in literature and the presence of combined genetic defects seems not to increase the risk of aHUS relapses. The incidence of ESRD is 46.7% which is quite similar to that in mutation-associated aHUS reported in published data (42%-60%) and appears different from the percentage of ESRD reported in other studies on anti-FH-associated aHUS (around 30%).[109] The worse outcome could depend by the presence of a high prevalence of mutations in our cohort (37%, see § 4.3.8) which in combination with the autoantibodies can increase the risk of renal failure.

A large proportion of patients treated with plasma exchange or infusion developed ESRD (43.5%) and this data is according to the literature where about 35% of cases show renal impairment despite the plasma exchange therapy. In this study, a

favourable outcome in patients treated with plasma in combination with steroids is not observed while in other studies plasma exchange and immunosuppression show a better outcome decreasing the percentage of patients who developed ESRD (14.3%).[109, 184] I wish to emphasize that my analysis of response to plasma and steroids is limited, because it is based on retrospective data; because of the rarity of anti-FH associated aHUS patients, it necessarily includes cases from several centres, where different plasma and immunosuppression protocols may have been used. Of note, none of our patients received anticomplement therapy.

Regard the kidney transplant, the limited number of our cases makes hard to make comparisons with published data. Of note, among 3 patients who underwent kidney transplant, a graft loss for aHUS recurrence was observed in only one who also carried a C3 mutation which is *per se* correlated to a high incidence of ESRD in patients with aHUS.[62]

In conclusion, in this chapter of the thesis, I have described the clinical features of a consistent group of aHUS patients with anti-FHs documenting that infections may have an important role in triggering full-blown disease. In addition, the concomitant presence of the homozygous *CFHR1*deletion and a disease onset between 4 and 12 years can predict with high sensitivity the presence of anti-FHs in a patients affected by aHUS. All these data could help the clinicians to identify patients with an autoimmune form of aHUS (especially when plasma therapy is already ongoing) and to adopt an appropriate therapy.

6 Analysis of class I and II MHC genes in patients with atypical hemolytic uremic syndrome and anti-factor H autoantibodies

6.1 Introduction

Atypical hemolytic uremic syndrome (aHUS) is usually determined by dysregulation of alternative pathway (AP) of complement system caused by genetic defects.[57] [62]

Anti-FH-mediated aHUS is associated with the deficiency of FHR1, a FH homologous protein.[107,] [123] The FH domains characterized by more similarities with FHR1 are SCR18, 19, and 20 and the main FH epitopes recognized by autoantibodies are included in this conserved region. Specifically, FH SCR18 has 3 residues that differ from those in FHR1 SCR3 (FH Tyr1058, Val1060, and Gln1076 that correspond to FHR1 His157, Leu159, and Gln175), FH SCR19 shows 100% of amino acid sequence identity with FHR1 SCR4, and FH SCR20 has only two residues different from FHR1 SCR5 (FH Ser1191 and Val1197 corresponding to FHR1 Leu290 and Ala296), as shown in Figure 6-1.

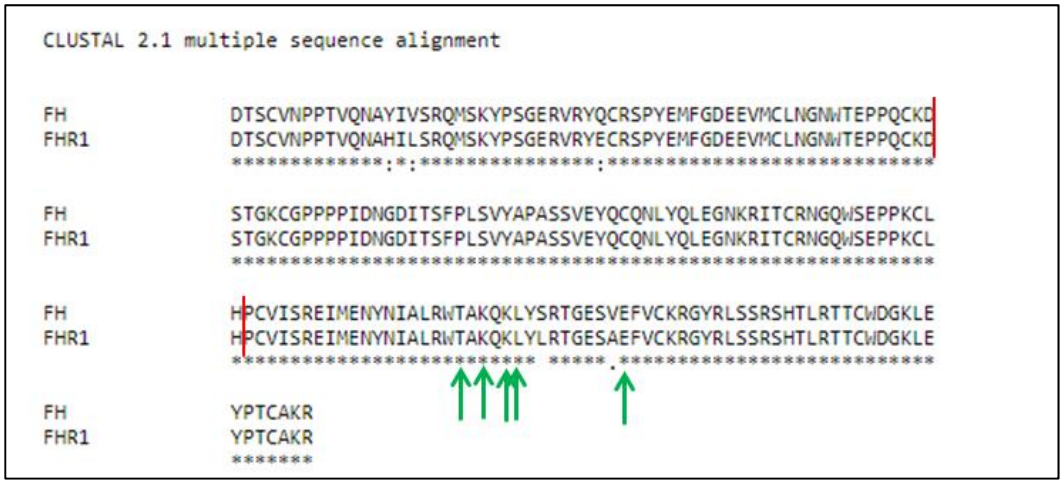

Figure 6-1: Clustal W alignment: the amino acid sequence alignment of the last 3 SCRs of FH (18-20) and of FHR1 (3-5) are shown.

* = indicates positions which have a single, fully conserved residue; : = indicates conservation between amino acid groups of strongly similar properties (score > 0.5 in the Gonnet PAM 250 matrix); . = indicates conservation between amino acid groups of weakly similar properties (score < 0.5 in the Gonnet PAM 250 matrix). Red lines indicate SCR borders. Green arrows indicated residues recognized by autoantibodies.

In most cases, the FHR1 deficiency is caused by homozygous deletion of *CFHR1* that is described in about 13% of aHUS cases (16.4% in our patients) and in about 5% of Caucasian healthy controls (data reported in chapter 4 of this thesis).[107]

Currently, the mechanisms involved in the loss of tolerance of the immune system against FH remain unknown. The deficiency of FHR1 observed in the large majority of aHUS patients with anti-FHs suggests its main role in autoimmune pathogenesis but it is not sufficient alone to cause autoantibody development. Genetic predisposition for most autoimmune diseases is polygenic as suggested by a large number of genome-wide association studies in which hundreds of risk loci

for autoimmune diseases have been identified.[151, 185] Some of these genetic variants are associated with several autoimmune diseases, suggesting that the causative genes influence general mechanisms of immune regulation and self-tolerance.[151] In autoimmune diseases, the strongest associations have been found with the MHC, indicating that some disease-associated MHC molecules may present self-antigens and activate auto-reactive T cells.[151]

There are 3 class I MHC genes (*A*, *B*, and *C*) and 3 class II MHC loci (*DR*, *DQ*, and *DP*). Each class II MHC locus contain *A* and *B* genes that encode for the alpha and beta chains of class II MHC heterodimer molecules.[134] The β chain of HLA-DR molecules is encoded by four *HLA-DRB* genes: *DRB1* and three additional paralogues (*DRB3*, *DRB4* and *DRB5)*. Each individual presents two haplotypes that generally contain *HLA-DRA1*, *HLA-DRB1* plus one additional *HLA-DRB* gene. All *HLA* genes are polymorphic and the allele nomenclature is based on serologic methods and molecular typing. All alleles have at least a four digit name, in which the first set of two digits corresponds to the serological antigen (or allele group) and the second set of two digits corresponds to a specific allele subtype. Alleles whose numbers differ in the second sets of digits differ in one or more nucleotide substitutions that determine amino acid changes in the MHC molecule (from hla.alleles.org).

Class I MHC molecules are expressed on all nucleated cells and are involved in the presentation of endogenous proteins, such as viral and tumor antigens, to CD8+ T cells. Instead, class II MHC molecules are expressed only on APCs (dendritic cells, B lymphocytes, macrophages, and a few other cells) and provide a system to display extracellular peptides derived from bacterial infections or not-

self proteins to CD4+ T cells. Both MHC class I and II are also involved in the maturation process of T lymphocytes in thymus. During the positive selection, T cells whose TCRs recognize self MHC molecules continue their differentiation and are committed to the CD4 or CD8 lineage based on the recognition of MHC class I rather than MHC class II. Then, during the negative selection, T cells whose TCRs strongly recognize self-peptide-MHC complex are deleted. These selections eliminate the potentially self-reactive T cells and ensure the self-tolerance.[134]

The peptide binding to MHC molecules depends on the peptide size and on non-covalent interactions by residues both in the peptides and the binding cleft of MHC molecules.[134] Since many residues in MHC peptide-binding cleft are polymorphic, the MHC alleles can bind several peptides with different affinity.[134] A weak affinity between self-peptides and MHC molecules can determine structural alterations in TCR-peptide-MHC complex that compromise its overall stability leading to the loss of immunological tolerance of self-antigens.[160, 161] My hypothesis is that a FH peptide could be not properly presented to CD4+ T cells in thymus in presence of specific MHC class II molecules causing the escape of auto-reactive CD4+ T cells during the negative selection. The FHR1 homologous peptide could be presented more efficiently by the same MHC class II molecules, preventing the generation of self-reactive T cells and as a consequence anti-FHs. Thus, according with this hypothesis, the FHR1 deficiency should increase the susceptibility for the autoimmune aHUS in patients carrying specific MHC class II variants.

In this chapter, I report the results of a case/control study in which I studied the MHC class II loci in 24 aHUS patients with anti-FHs and in 64 healthy controls carrying the homozygous deletion of *CFHR1,* here defined as "super controls". I also included MHC class I analyses since MHC class I alleles are also reported to be implicated in the pathogenesis of autoimmunity in many diseases and I could not exclude at all their involvement. In this study I report *DRB1*11:04* as a possible candidate HLA allele that could predispose to anti-FH-mediated aHUS.

6.2 Specific aims

To evaluate the presence of *HLA-A*, *B*, *C*, *DRB1*, *DRB3*, *DRB4*, *DRB5*, *DQA1*, *DQB1*, *DPA1*, and *DPB1* risk alleles in aHUS patients with anti-FH antibodies *versus* healthy controls both carrying the homozygous *CFHR1* deletion.

To evaluate the OR for each risk HLA allele.

To evaluate the presence of risk allele in aHUS patients positive for anti-FHs who do not carry the homozygous *CFHR1* deletion.

To test *in silico* the affinity of FHR1/FH peptides with the newly identified predisposing MHC molecules.

6.3 Results

6.3.1 Power analysis

I planned a study with 24 aHUS cases with anti-FH antibodies and 64 healthy controls both carrying the FHR1 deficiency. Data obtained in chapter 4 of this thesis showed that 1 out of 13 controls (7%) with the homozygous *CFHR1* deletion was positive for anti-FHs. In literature the prevalence of anti-FH antibodies in healthy controls was reported to be about 1-4%. Therefore, I arbitrarily decided to consider 4% the estimated probability for a healthy control carrying the homozygous *CFHR1* deletion to develop anti-FHs. Considering that the probability to develop anti-FHs among aHUS patients with the homozygous *CFHR1* deletion is about 48% (data reported in chapter 4 of this thesis), I could reject the null hypothesis that the probability of anti-FH antibodies in cases and controls was equal with a power of 0.995. The Type I error probability associated with this test of this null hypothesis was 0.05. I used Chi-squared test or Fisher's exact test to evaluate this null hypothesis.

6.3.2 HLA study

HLA-A, *B*, *C*, *DRB1*, *DRB3*, *DRB4*, *DRB5*, *DQA1*, *DQB1*, *DPA1*, and *DPB1* allelic frequencies (obtained considering the incidence of one allele/total allele number) and carrier frequencies (obtained from the number of individuals with at least one specific allele/total individual number) were established in 24 aHUS cases and 64 controls (not all analyzed for all *HLA* genes) carrying the FHR1 deficiency. About half of patients were Italians (n = 10) but there were also 1 Portuguese, 3 Caucasian Americans, 1 German, 2 Serbian, 1 Israel Jewish, 1

Japanese, 1 Bulgarian, 1 Iranian, 2 Polish, and 1 Dutch. All cases carried the FHR1 deficiency. The healthy controls were mostly Italian (n = 57) but I also included in the analyses 3 Israel Jews and 4 Iranians.

MHC class I

The results of the comparison of *HLA-A* allele and carrier frequencies between aHUS and healthy controls are shown in Table 6-1 (2 digits) and Table 2 (4 digits). Sixteen alleles were detected and the most common in both aHUS cases and controls were the 01, 02, and 24 alleles. The 03 allele appeared also common in controls but not in aHUS cases despite no statistical difference was evident comparing the allele frequencies in patient and control groups (Table 6-1).

HLA-A Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=108)			**Pt** (n=24)		**Ctr** (n=54)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	10	0.21	12	0.11	0.174	9	0.38	12	0.22	0.260
02	9	0.19	26	0.24	0.598	8	0.33	22	0.41	0.713
03	2	0.04	12	0.11	0.229	2	0.08	11	0.20	0.323
11	3	0.06	8	0.07	1.000	3	0.13	7	0.13	1.000
23	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
24	9	0.19	14	0.13	0.486	7	0.29	14	0.26	0.983
25	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
26	3	0.06	3	0.03	0.373	3	0.13	3	0.06	0.365
29	2	0.04	4	0.04	1.000	2	0.08	4	0.07	1.000
30	1	0.02	5	0.05	0.667	1	0.04	5	0.09	0.660
31	1	0.02	8	0.07	0.277	1	0.04	7	0.13	0.423
32	5	0.10	5	0.05	0.286	4	0.17	5	0.09	0.445
33	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
68	1	0.02	4	0.04	1.000	1	0.04	4	0.07	1.000
69	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
80	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000

Table 6-1: Statistical comparison of *HLA-A* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 54 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

Twenty-two different alleles were detected considering the 4 digit resolution and the most frequent were 01:01, 02:01, and 24:02 alleles in both groups. The 03:01 was present in 11% of healthy controls *versus* 4% of aHUS cases but the prevalence was not significantly different between the two groups. The 29:01 allele has been identified only in one patient and not in controls (Table 6-2). Overall, *HLA-A* allele and carrier frequencies were comparable between patients and controls and no significant association was found in both 2 and 4 digit resolutions.

HLA-A Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=108)			**Pt** (n=24)		**Ctr** (n=54)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	10	0.21	12	0.11	0.174	9	0.38	12	0.22	0.260
02:01	7	0.15	25	0.23	0.314	6	0.25	21	0.39	0.351
02:05	2	0.04	1	0.01	0.224	2	0.08	1	0.02	0.223
03:01	2	0.04	12	0.11	0.229	2	0.08	11	0.20	0.323
11:01	3	0.06	8	0.07	1.000	3	0.13	7	0.13	1.000
23:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
24:02	9	0.19	12	0.11	0.300	7	0.29	12	0.22	0.709
24:03	0	0.00	2	0.02	1.000	0	0.00	2	0.04	1.000
25:01	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
26:01	3	0.06	3	0.03	0.373	3	0.13	3	0.06	0.365
29:01	1	0.02	0	0.00	0.308	1	0.04	0	0.00	0.308
29:02	1	0.02	4	0.04	1.000	1	0.04	4	0.07	1.000
30:01	1	0.02	4	0.04	1.000	1	0.04	4	0.07	1.000
30:02	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
31:01	1	0.02	8	0.07	0.277	1	0.04	7	0.13	0.423
32:01	5	0.10	5	0.05	0.286	4	0.17	5	0.09	0.445
33:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
33:03	1	0.02	1	0.01	0.522	1	0.04	1	0.02	0.523
68:01	0	0.00	3	0.03	0.553	0	0.00	3	0.06	0.549
68:02	1	0.02	1	0.01	0.522	1	0.04	1	0.02	0.523
69:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
80:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000

Table 6-2: Statistical comparison of *HLA-A* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 54 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

In Table 6-3 and 6-4 I report the results of the association study regard *HLA-B* alleles (2 and 4 digits, respectively). Twenty-three 2 digit alleles were detected and the most frequent in cases and controls was the 35 allele. The 51 allele was more represented in healthy subjects than in aHUS cases but the allelic frequency difference between the two groups was not significant (aHUS 4% *versus* controls 14%, ns, Table 6-3).

HLA-B Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=106)			**Pt** (n=24)		**Ctr** (n=53)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
07	2	0.04	4	0.04	1.000	2	0.08	4	0.08	1.000
08	4	0.08	5	0.05	0.461	4	0.17	5	0.09	0.448
13	3	0.06	5	0.05	0.705	3	0.13	4	0.08	0.671
14	1	0.02	3	0.03	1.000	1	0.04	3	0.06	1.000
15	3	0.06	3	0.03	0.376	3	0.13	3	0.06	0.369
18	4	0.08	5	0.05	0.461	3	0.13	5	0.09	0.699
27	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
35	9	0.19	22	0.21	0.944	9	0.38	20	0.38	0.815
37	0	0.00	2	0.02	1.000	0	0.00	2	0.04	1.000
38	4	0.08	7	0.07	0.740	3	0.13	7	0.13	1.000
39	2	0.04	2	0.02	0.589	2	0.08	2	0.04	0.585
40	2	0.04	3	0.03	0.647	2	0.08	2	0.04	0.585
41	1	0.02	1	0.01	0.528	1	0.04	1	0.02	0.529
44	5	0.10	9	0.08	0.675	4	0.17	9	0.17	1.000
45	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
47	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
49	0	0.00	5	0.05	0.325	0	0.00	5	0.09	0.317
50	2	0.04	1	0.01	0.229	2	0.08	1	0.02	0.551
51	2	0.04	15	0.14	0.095	2	0.08	14	0.26	0.127
53	1	0.02	0	0.00	0.312	1	0.04	0	0.00	0.312
55	0	0.00	6	0.06	0.177	0	0.00	5	0.09	0.317
57	2	0.04	1	0.01	0.229	2	0.08	1	0.02	0.228
58	0	0.00	3	0.03	0.552	0	0.00	3	0.06	0.548

Table 6-3: Statistical comparison of *HLA-B* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 53 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

The analysis was repeated considering 4 digit resolution and 34 alleles have been detected. The most common were the 35:02 in aHUS cases and the 35:01 and 51:01 in controls while the 07:05, 15:09, 50:02, and 53:01 alleles were found only in 4 different aHUS patients and not in controls (Table 6-4). Overall, no statistically significant difference was observed in allele and carrier frequencies between the case and the control groups.

HLA-B Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=106)			**Pt** (n=24)		**Ctr** (n=53)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
07:02	1	0.02	4	0.04	1.000	1	0.04	4	0.08	1.000
07:05	1	0.02	0	0.00	0.312	1	0.04	0	0.00	0.312
08:01	4	0.08	5	0.05	0.461	4	0.17	5	0.09	0.448
13:02	3	0.06	5	0.05	0.705	3	0.13	4	0.08	0.671
14:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
14:02	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
15:01	2	0.04	2	0.02	1.000	2	0.08	2	0.04	0.585
15:09	1	0.02	0	0.00	0.312	1	0.04	0	0.00	0.312
15:24	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
18:01	4	0.08	5	0.05	0.461	3	0.13	5	0.09	0.699
27:05	1	0.02	2	0.02	1.000	1	0.04	2	0.04	1.000
35:01	3	0.06	10	0.09	0.756	3	0.13	10	0.19	0.744
35:02	5	0.10	5	0.05	0.287	5	0.21	4	0.08	0.127
35:03	1	0.02	6	0.06	0.436	1	0.04	6	0.11	0.424
35:158	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
37:01	0	0.00	2	0.02	1.000	0	0.00	2	0.04	1.000
38:01	4	0.08	7	0.07	0.740	3	0.13	7	0.13	1.000
39:01	2	0.04	2	0.02	0.589	2	0.08	2	0.04	0.585
40:01	2	0.04	2	0.02	0.589	2	0.08	2	0.04	0.585
40:02	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
41:01	1	0.02	1	0.01	0.528	1	0.04	1	0.02	0.529
44:03	2	0.04	3	0.03	0.647	2	0.08	3	0.06	0.644
45:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
47:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
49:01	0	0.00	5	0.05	0.325	0	0.00	5	0.09	0.317
50:01	1	0.02	1	0.01	0.528	1	0.04	1	0.02	0.529
50:02	1	0.02	0	0.00	0.312	1	0.04	0	0.00	0.312
51:01	2	0.04	12	0.11	0.228	2	0.08	12	0.23	0.203
51:07	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
51:08	0	0.00	2	0.02	1.000	0	0.00	2	0.04	1.000
53:01	1	0.02	0	0.00	0.312	1	0.04	0	0.00	0.312
55:01	0	0.00	6	0.06	0.178	0	0.00	5	0.09	0.317
57:01	2	0.04	1	0.01	0.229	2	0.08	1	0.02	0.228
58:01	0	0.00	3	0.03	0.552	0	0.00	3	0.06	0.548

Table 6-4: Statistical comparison of *HLA-B* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 53 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

The comparison of *HLA-C* allele and carrier frequencies between cases and controls is reported in Tables 6-5 and 6-6.

HLA-C Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=110)			**Pt** (n=24)		**Ctr** (n=55)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	0	0.00	8	0.07	0.107	0	0.00	7	0.13	0.094
02	1	0.02	5	0.05	0.668	1	0.04	5	0.09	0.661
03	4	0.08	11	0.10	1.000	4	0.17	11	0.20	1.000
04	9	0.19	20	0.18	0.890	9	0.38	19	0.35	0.997
05	3	0.06	6	0.05	1.000	2	0.08	6	0.11	1.000
06	7	0.15	10	0.09	0.456	7	0.29	8	0.15	0.226
07	11	0.23	20	0.18	0.637	10	0.42	20	0.36	0.846
08	1	0.02	3	0.03	1.000	1	0.04	3	0.05	1.000
12	7	0.15	12	0.11	0.699	6	0.25	11	0.20	0.842
14	2	0.04	5	0.05	1.000	2	0.08	5	0.09	1.000
15	2	0.04	3	0.03	0.640	2	0.08	3	0.05	1.000
16	1	0.02	7	0.06	0.437	1	0.04	7	0.13	0.423

Table 6-5: Statistical comparison of *HLA-C* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 55 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

Twelve alleles were found for *HLA-C* gene considering the 2 digit resolution. The 04, 06, 07, and 12 were the more abundant in both case and control groups (Table 6-5). Repeating the analysis with a higher resolution, among 21 alleles, the most common were 04:01, 06:02, 07:01, and 12:03 in both cases and controls. The 14:03 and 15:05 alleles were present exclusively in aHUS patients. No significant differences were found in allele and carrier frequencies comparing cases and controls (Table 6-6).

HLA-C Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=110)			**Pt** (n=24)		**Ctr** (n=55)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:02	0	0.00	8	0.07	0.107	0	0.00	7	0.13	0.094
02:02	1	0.02	5	0.05	0.668	1	0.04	5	0.09	0.661
03:02	0	0.00	3	0.03	0.554	0	0.00	3	0.05	0.315
03:03	2	0.04	6	0.05	1.000	2	0.08	6	0.11	1.000
03:04	2	0.04	2	0.02	0.585	2	0.08	2	0.04	0.581
04:01	9	0.19	20	0.18	0.890	9	0.38	19	0.35	0.997
05:01	3	0.06	6	0.05	1.000	3	0.13	6	0.11	1.000
06:02	7	0.15	9	0.08	0.347	7	0.29	8	0.15	0.226
07:01	6	0.13	14	0.13	0.825	6	0.25	14	0.25	0.811
07:02	4	0.08	5	0.05	0.456	4	0.17	5	0.09	0.443
07:04	1	0.02	1	0.01	0.517	1	0.04	1	0.02	0.518
08:02	1	0.02	3	0.03	1.000	1	0.04	3	0.05	1.000
12:02	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
12:03	7	0.15	11	0.10	0.574	6	0.25	11	0.20	0.842
14:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
14:02	1	0.02	4	0.04	1.000	1	0.04	4	0.07	1.000
14:03	1	0.02	0	0.00	0.304	1	0.04	0	0.00	0.304
15:02	1	0.02	3	0.03	1.000	1	0.04	3	0.05	1.000
15:05	1	0.02	0	0.00	0.304	1	0.04	0	0.00	0.304
16:01	1	0.02	3	0.03	1.000	1	0.04	3	0.05	1.000
16:02	0	0.00	4	0.04	0.315	0	0.00	4	0.07	0.308

Table 6-6: Statistical comparison of *HLA-C* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 55 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction**.**

MHC class II

Thirteen *HLA-DRB1* alleles were found, of whom the most frequent were the 03, 07, 11, 13, and 15 in aHUS cases and the 04, 07, 11, and 13 in controls but no significant differences were present in the allelic and/or carrier frequencies between the two groups (Table 6-7).

HLA-DRB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=128)			**Pt** (n=24)		**Ctr** (n=64)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	4	0.08	10	0.08	1.000	4	0.17	8	0.13	0.729
03	7	0.15	9	0.07	0.209	6	0.25	9	0.14	0.370
04	4	0.08	13	0.10	1.000	4	0.17	13	0.20	1.000
07	6	0.13	13	0.10	0.862	6	0.25	12	0.19	0.726
08	0	0.00	4	0.03	0.576	0	0.00	4	0.06	0.571
09	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
10	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
11	12	0.25	32	0.25	0.845	10	0.42	28	0.44	0.948
12	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
13	7	0.15	18	0.14	0.877	7	0.29	16	0.25	0.902
14	1	0.02	7	0.05	0.450	1	0.04	6	0.09	0.689
15	5	0.10	9	0.07	0.533	5	0.21	8	0.13	0.330
16	2	0.04	8	0.06	0.730	2	0.08	7	0.11	1.000

Table 6-7: Statistical comparison of HLA-*DRB1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 64 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

To go deeper into the results of *DRB1*, I repeated the case-control study considering the 4 digit resolution (Table 6-8). Thirty-three different alleles have been detected and the most common in patients were the 03:01, 07:01, 11:04, and 15:01 alleles while in controls were the 07:01 and 11:01 alleles. The 01:03, 04:08 and 13:11 alleles were found only in 3 different Italian patients and not in

controls. Interestingly, I found that carrier frequency of *DRB1**11:04 allele was higher in aHUS patients compared to controls (38% *versus* 13%, p-value, unadjusted = 0.019). The significance cut-off after the Bonferroni correction for multiplying tests (the number of alleles for each MHC gene) was set at 0.002 for *DRB1* and thus, the association with 11:04 allele did not reach statistical significance. Logistic regression showed that the presence of HLA-*DRB1**11:04 allele in individuals carriers of the homozygous *CFHR1* deletion increased the probability to be an aHUS patient with anti-FH antibodies (OR = 4.2, 95% CI [1.384-12.742], p-value, unadjusted = 0.011).

HLA-DRB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=128)			**Pt** (n=24)		**Ctr** (n=64)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	3	0.06	6	0.05	0.706	3	0.13	5	0.08	0.678
01:02	0	0.00	4	0.03	0.576	0	0.00	3	0.05	0.559
01:03	1	0.02	0	0.00	0.273	1	0.04	0	0.00	0.273
03:01	7	0.15	9	0.07	0.209	6	0.25	9	0.14	0.370
04:01	2	0.04	3	0.02	0.615	2	0.08	3	0.05	0.611
04:02	0	0.00	3	0.02	0.563	0	0.00	3	0.05	0.559
04:03	0	0.00	3	0.02	0.563	0	0.00	3	0.05	0.559
04:04	0	0.00	3	0.02	0.563	0	0.00	3	0.05	0.559
04:05	1	0.02	1	0.01	0.472	1	0.04	1	0.02	0.473
04:08	1	0.02	0	0.00	0.273	1	0.04	0	0.00	0.273
07:01	6	0.13	13	0.10	0.862	6	0.25	12	0.19	0.726
08:01	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
08:02	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
08:04	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
09:01	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
10:01	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
11:01	2	0.04	17	0.13	0.104	2	0.08	16	0.25	0.136
11:02	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
11:03	1	0.02	3	0.02	1.000	1	0.04	3	0.05	1.000
11:04	9	0.19	10	0.08	0.070	9	0.38	8	0.13	**0.019**
11:24	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
12:01	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
13:01	2	0.04	10	0.08	0.734	2	0.08	10	0.16	0.500
13:02	3	0.06	5	0.04	0.685	3	0.13	4	0.06	0.385
13:03	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
13:05	1	0.02	1	0.01	0.472	1	0.04	1	0.02	0.473
13:11	1	0.02	0	0.00	0.273	1	0.04	0	0.00	0.273
14:01	1	0.02	5	0.04	1.000	1	0.04	5	0.08	1.000
14:04	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
14:54	0	0.00	1	0.01	1.000	0	0.00	1	0.02	1.000
15:01	5	0.10	7	0.05	0.410	5	0.21	7	0.11	0.392
15:02	0	0.00	2	0.02	1.000	0	0.00	2	0.03	1.000
16:01	2	0.04	8	0.06	0.730	2	0.08	7	0.11	1.000

Table 6-8: Statistical comparison of *HLA-DRB1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 64 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

I have also performed the case/control study considering the *DRB3*, *DRB4*, and *DRB5* genes and the results are reported in Tables 6-9, 6-10, and 6-11, respectively. In this analysis I only considered the results of higher resolution

analysis because a limited number of alleles were present in *DRB3-4-5* genes due to a lower variability compared to the main *DRB1* gene. I observed the presence of *DRB3* gene in 21 aHUS cases (87.5%) *versus* 23 controls (74.2%), of *DRB4* in 10 aHUS cases (41.7%) *versus* 12 controls (38.7%), and of *DRB5* in 6 aHUS cases (25%) *versus* 7 controls (22.6%) and these differences were not statistically significant. The comparison of *DRB3*, *DRB4*, and *DRB5* allele and carrier frequencies between cases and controls did not reveal any significant associations of these additional *DRB* genes with the risk of aHUS with anti-FHs.

HLA-DRB3 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	4	0.08	6	0.10	1.000	4	0.17	6	0.19	1.000
02:01	1	0.02	0	0.00	0.436	1	0.04	0	0.00	0.436
02:02	19	0.40	24	0.39	0.918	15	0.67	19	0.61	0.851
03:01	3	0.06	2	0.03	0.651	3	0.13	1	0.03	0.307
absence	21	0.44	30	0.48	0.771	17	0.75	22	0.71	0.773

Table 6-9: Statistical comparison of *HLA-DRB3* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

HLA-DRB4 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	10	0.21	16	0.26	0.702	9	0.38	12	0.39	0.851
absence	38	0.79	46	0.74	0.702	23	0.96	27	0.87	0.378

Table 6-10: Statistical comparison of *HLA-DRB4* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction**.**

HLA-DRB5 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	5	0.10	3	0.10	0.293	5	0.21	3	0.10	0.276
01:02	0	0.00	1	0.03	1.000	0	0.00	1	0.03	1.000
02:02	2	0.04	4	0.13	0.694	2	0.08	4	0.13	0.686
absence	41	0.85	54	0.97	0.980	23	0.96	30	0.97	1.000

Table 6-11: Statistical comparison of *HLA-DRB5* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

The comparison of *DQA1* allele and carrier frequencies between aHUS and healthy controls is shown in Tables 6-12 and 6-13 (2 and 4 digits, respectively). I found 5 alleles in *DQA1* gene and the more abundant were the 01 and 05 in both patients and control groups. Considering the higher typing resolution there were 7 alleles and the most frequent were the 01:01, 01:02, 02:01, and 05:01 in aHUS patients and 01:02, 02:01, 03:01, and 05:01 in controls. All alleles were equally represented in both groups.

HLA-DQA1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	17	0.35	20	0.32	0.885	14	0.58	17	0.55	0.988
02	6	0.13	8	0.13	0.822	6	0.25	7	0.23	0.912
03	4	0.08	8	0.13	0.546	4	0.17	8	0.26	0.519
04	0	0.00	3	0.05	0.255	0	0.00	3	0.10	0.248
05	21	0.44	23	0.37	0.610	17	0.71	18	0.58	0.488

Table 6-12: Statistical comparison of *HLA-DQA1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

HLA-DQA1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	5	0.10	4	0.06	0.500	5	0.21	4	0.13	0.482
01:02	10	0.21	9	0.15	0.539	8	0.33	8	0.26	0.756
01:03	2	0.04	7	0.11	0.294	2	0.08	7	0.23	0.271
02:01	6	0.13	8	0.13	0.822	6	0.25	7	0.23	0.912
03:01	4	0.08	8	0.13	0.546	4	0.17	8	0.26	0.519
04:01	0	0.00	3	0.05	0.255	0	0.00	3	0.10	0.248
05:01	21	0.44	23	0.37	0.610	17	0.71	18	0.58	0.488

Table 6-13: Statistical comparison of *HLA-DQA1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

The *DQB1* allele and carrier frequencies in aHUS and in healthy controls are shown in Tables 14 and 15 (2 and 4 digits, respectively). The lower resolution showed 5 *DQB1* alleles of whom the 02, 03, 05, and 06 were the most abundant in patients and controls (Table 6-14).

HLA-DQB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=128)			**Pt** (n=24)		**Ctr** (n=64)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
02	10	0.21	24	0.19	0.922	8	0.33	22	0.34	0.872
03	21	0.44	49	0.38	0.626	16	0.67	40	0.63	0.910
04	0	0.00	4	0.03	0.576	0	0.00	4	0.06	0.571
05	7	0.15	29	0.23	0.331	7	0.29	25	0.39	0.541
06	10	0.21	22	0.17	0.735	9	0.38	20	0.31	0.764

Table 6-14: Statistical comparison of *HLA-DRQ1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 64 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

By deepening the analysis with 4 digits, 15 alleles were observed. The most common *DQB1* alleles were the 02:01, 03:01, and 06:02 in aHUS patients and the 02:01, 03:01, 03:02, and 05:01 in controls (Table 6-15). The 06:04 allele was found in 3 aHUS cases and not in controls (p-value, unadjusted = 0.031). The significance was lost after Bonferroni correction for multiple tests (significance threshold = 0.003). One aHUS patients carrying *DQB1**06:04 allele also showed the *DRB1**11:04 allele.

HLA-DQB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=100)			**Pt** (n=24)		**Ctr** (n=50)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
02:01	10	0.21	15	0.15	0.514	8	0.33	14	0.28	0.843
02:02	0	0.00	4	0.04	0.305	0	0.00	4	0.08	0.297
03:01	15	0.31	28	0.28	0.830	13	0.54	24	0.48	0.804
03:02	2	0.04	11	0.11	0.224	2	0.08	11	0.22	0.200
03:03	3	0.06	1	0.01	0.100	3	0.13	1	0.02	0.097
03:04	1	0.02	0	0.00	0.324	1	0.04	0	0.00	0.243
04:02	0	0.00	4	0.04	0.305	0	0.00	4	0.08	0.297
05:01	4	0.08	11	0.11	0.774	4	0.17	10	0.20	1.000
05:02	2	0.04	5	0.05	1.000	2	0.08	5	0.10	1.000
05:03	1	0.02	6	0.06	0.428	1	0.04	5	0.10	0.657
06:01	0	0.00	3	0.03	0.551	0	0.00	3	0.06	0.546
06:02	5	0.10	3	0.03	0.113	5	0.21	3	0.06	0.103
06:03	2	0.04	7	0.07	0.719	2	0.08	7	0.14	0.709
06:04	3	0.06	0	0.00	**0.033**	3	0.13	0	0.00	**0.031**
06:09	0	0.00	2	0.02	1.000	0	0.00	1	0.02	1.000

Table 6-15: Statistical comparison of *HLA-DQB1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 50 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

The results of comparison of *DPA1* allele and carrier frequencies between aHUS and healthy controls is reported in Tables 16 and 17 (low and high resolution, respectively). The 01:03 allele was the most frequent in both aHUS patients and controls and no differences were evident between the two groups.

HLA-DPA1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	38	0.79	53	0.85	0.539	24	1.00	30	0.97	1.000
02	10	0.21	9	0.15	0.539	10	0.42	8	0.26	0.340

Table 6-16: Statistical comparison of *HLA-DPA1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

HLA-DPA1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	*P*-value	n	freq	n	freq	*P*-value
01:03	38	0.79	52	0.84	0.700	24	1.00	30	0.97	0.897
01:XXX	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000
02:01	9	0.19	7	0.11	0.408	9	0.38	6	0.19	0.233
02:02	1	0.02	2	0.03	1.000	1	0.04	2	0.06	1.000

Table 6-17: Statistical comparison of *HLA-DPA1* allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction. DPA1*01:XXX was an allele not discriminated.

Regarding *HLA-DPB1*, 13 alleles have been observed considering 2 digit resolution. The alleles more frequent in patients and controls were the 02, 03, and 04. At 4 digit resolution, the 04 allele included 04:01 and 04:02 alleles that were represented in equal measure in case and control groups. The 05:01 and 09:01 alleles were present in only 2 different aHUS patients and in not in controls. Overall *DPB1* allele and carrier frequencies showed no differences between aHUS patients and controls.

HLA-DPB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01	2	0.04	1	0.02	0.579	2	0.08	1	0.03	0.575
02	8	0.17	11	0.18	0.915	7	0.29	11	0.35	0.837
03	5	0.10	8	0.13	0.772	5	0.21	7	0.23	1.000
04	25	0.52	34	0.55	0.925	21	0.88	27	0.87	1.000
05	2	0.04	0	0.00	0.188	2	0.08	0	0.00	0.186
09	1	0.02	0	0.00	0.436	1	0.04	0	0.00	0.436
10	3	0.06	2	0.03	0.651	3	0.13	2	0.06	0.643
13	1	0.02	1	0.02	1.000	1	0.04	1	0.03	1.000
14	1	0.02	2	0.03	1.000	1	0.04	2	0.06	1.000
17	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000
19	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000
29	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000

Table 6-18: Statistical comparison of HLA-DPB1 allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 2 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

HLA-DPB1 Allele	Allele					Carrier				
	Pt (n=48)		**Ctr** (n=62)			**Pt** (n=24)		**Ctr** (n=31)		
	n	freq	n	freq	***P*-value**	n	freq	n	freq	***P*-value**
01:01	2	0.04	1	0.02	0.579	2	0.08	1	0.03	0.575
02:01	8	0.17	11	0.18	0.915	7	0.29	11	0.35	0.837
03:01	5	0.10	8	0.13	0.772	5	0.21	7	0.23	1.000
04:01	17	0.35	26	0.42	0.619	15	0.63	22	0.71	0.708
04:02	8	0.17	8	0.13	0.778	7	0.29	7	0.23	0.807
05:01	2	0.04	0	0.00	0.188	2	0.08	0	0.00	0.186
09:01	1	0.02	0	0.00	0.436	1	0.04	0	0.00	0.436
10:01	3	0.06	2	0.03	0.651	3	0.13	2	0.06	0.643
13:01	1	0.02	1	0.02	1.000	1	0.04	1	0.03	1.000
14:01	1	0.02	2	0.03	1.000	1	0.04	2	0.06	1.000
17:01	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000
19:01	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000
29:01	0	0.00	1	0.02	1.000	0	0.00	1	0.03	1.000

Table 6-19: Statistical comparison of HLA-DPB1 allele and carrier frequencies in 24 aHUS patients with anti-FHs and 31 healthy controls both carriers of FHR1 deficiency.

Alleles were defined with 4 digits. P-values obtained from Chi-square test (or Fisher's exact test when the number of individuals for each category was five or less) were reported without Bonferroni correction.

In conclusion, the case/control study showed that *DRB1**11:04 and the *DQB1**06:04 alleles were the only HLA alleles with a trend association with aHUS and anti-FHs but after the Bonferroni correction the significance was lost for both.

Thus, I performed further analyses on these two *HLA* alleles since the loss of significance could be linked to the low number of patients and controls that was a limitation of this study. I investigated the presence of *DRB1**11:04 and *DQB1**06:04 alleles in 4 aHUS patients positive for anti-FHs and without the FHR1 deficiency and in an healthy control presenting anti-FHs and the homozygous deletion of *CFHR1,* for whom the *HLA* loci were analyzed in 4 digit resolution. Only 1 aHUS patient from Turkey with anti-FHs resulted to carry the *HLA-DRB1**11:04 allele. Interestingly, I also analyzed an Italian patient affected by Dense Deposit Disease, a rare kidney disease in which the AP of complement system is involved, with anti-FHs and the homozygous *CFHR1* deletion, and he showed the *DRB1**11:04 allele. None of the above subjects was a carrier of the *DQB1**06:04 allele.

The *DRB1**11:04 allele has been reported to have carrier frequency ranges from 2% to about 30% in different populations (www.allelefrequencies.net). In Table 6-20, I reported the carrier frequencies of *DRB1**11:04 allele in our aHUS patients and in healthy controls according to specific populations to which patients belonged. I also considered the cumulative carrier frequencies of *DRB1**11:04 allele in European and not European patient and control populations, and the cumulative carrier frequencies in the overall patient and control populations.

Populations	aHUS			Controls		
	n	**carriers**	**freq**	**n**	**carriers**	**freq**
Italy	*11*	*4*	*0.36*	*5,848*	*1,525*	*0.26*
Netherlands	1	0		1,816	98	0.05
Poland	2	1		20,952	1,752	0.08
Portugal	1	1		275	15	0.05
Bulgaria	1	0		55	17	0.31
Serbia	2	1		na	na	na
Germany	1	0		48,662	3,394	0.07
Overall European (Italians excluded)	*8*	*3*	*0.38*	*71,760*	*5,276*	*0.07*
Turkey	1	1		250	31	0.12
Iran	1	0		na	na	na
Israel (Jews)	1	0		4,837	1,583	0.33
Japan	1	0		195	0	0.00
American (Caucasian)	3	2		1,304,545	76,227	0.06
Argentine	2	0		466	29	0.06
Overall Not European	*9*	*3*	*0.33*	*1,310,293*	*77,871*	*0.06*
Overall patients	***28***	***10***	***0.36***	***1,387,901***	***84,672***	***0.06***

Table 6-20: Comparison of *HLA-DRB1*11:04* carrier frequencies in 28 aHUS patients with anti-FHs of our cohort and in some healthy controls reported on www.allelefrequencies.net.

The reported populations were those belonging to at least one aHUS patient. In the "overall" lines were shown the cumulative frequencies in European (Italians excluded) and not-European patients and controls. The allele frequency for each control population was obtained from the frequency mean reported on allele.frequencies.net ("Italy Bergamo", "Italy North pop3", "Italy pop4", "Italy pop5", "Italy Rome", "Netherlands", "Netherlands Leiden", "Netherlands UMCU", "Poland", "Poland Wielkopolska Region", "Poland DKMS", "Portugal Azores Terceira Island", "Portugal Center", "Portugal North", "Portugal South", "Bulgaria", "Serbia", "Germany pop 3", "Germany pop 6", "Germany pop 8", "Turkey pop 1", "Iran", "Israel Ashkenazi Jews", "Israel Ashkenazi Jews pop 2", "Israel Non-Ashkenazi Jews", "Japan Hyogo Osaka and Nagano", "USA Caucasian", "USA NMDP European Caucasian", "Argentina Buenos Aires").

The *DRB1**11:04 allele was found to be more frequent in aHUS patients with anti-FH compared to controls reported on allele.frequencies.net database. Considering the cumulative frequency in the overall populations, the difference was statistically significant (36% aHUS *versus* 6% controls, Chi-square test, p-value = 7.65×10^{-10}). Of note, this comparison showed that the *DRB1**11:04 allele is more abundant in Italians controls (26%) compared to other analyzed European (7%) and not-European populations (6%). Moreover, the 11:04 allele resulted

quite common in Bulgaria and in Jewish populations, but overall both European and not-European populations showed a carrier frequency < 13%.

To increase the number of healthy controls, I have used 228 "Not Finnish-European" healthy controls from 1000 Genomes project available on ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/working/20140725_hla_genotypes which were typed by Sanger sequencing on *HLA* genes in 4 digit resolution.[186] Sixteen individuals were carriers of the *DRB1**11:04 (7%) and I selected the controls that also carried the homozygous *CFHR3-CFHR1* deletion that is the most common polymorphism that determine the lack of *CFHR1* (n=8).[42] Two out of 8 controls with the homozygous deletion of *CFHR1* were carriers of *DRB1**11:04 and were Italians. Thus, overall, I observed 9/24 (38%) aHUS cases *versus* 10/72 (14%) controls carrying the homozygous deletion of *CFHR1* and the *DRB1**11:04 (Chi-square test, p-value = 0.025).

6.3.3 *In silico* analysis

NetMHCIIpan Server is available online software to predict the affinity between MHC class II molecules and binding peptides. I performed an affinity analysis with NetMHCIIpan 3.0 considering FH C terminus peptides containing the residues known to be involved in the anti-FH binding. In this region, 2 amino acidic differences were present between FH and FHR1 sequences (FH= Ser1191and Val11197 and FHR1= Leu290 and Ala296). The analysis showed that RWTAKQKLY**S**RTGES and WTAKQKLY**S**RTGES**V** FH peptides have a low affinity for the DRB1*11:04 molecules (Table 6-21).

FH Peptides	Binding core register	Position	Affinity(nM)	BindingLevel
LRWTAKQKLY**S**RTGE	WTAKQKLY**S**	2	37.83	SB
RWTAKQKLY**S**RTGES	WTAKQKLY**S**	1	77.99	WB
WTAKQKLY**S**RTGES**V**	WTAKQKLY**S**	0	131.24	WB
TAKQKLY**S**RTGES**V**E	LY**S**RTGES**V**	5	298.78	WB
AKQKLY**S**RTGES**V**EF	LY**S**RTGES**V**	4	269.69	WB
KQKLY**S**RTGES**V**EFV	LY**S**RTGES**V**	3	233.58	WB
QKLY**S**RTGES**V**EFVC	LY**S**RTGES**V**	2	493.67	WB

Table 6-21: Binding prediction of MHC class II DRB1*11:04 molecule for C-terminus FH-derived peptides (15 mer) determined by NetMHCIIpan 3.0 available online.

The affinity (nM) represents the IC50 values obtained during the analysis. SB = strong binder, WB = weak binder.

The same analysis performed with the homologous RWTAKQKLY**L**RTGES and WTAKQKLY**L**RTGES**A** FHR1 peptides predicted a higher affinity for DRB1*11:04 molecules than FH peptides (Table 6-22). Interestingly, the C terminus FH derived peptides showed overall a lower affinity for DRB1*11:04 molecules than the C terminus FHR1 peptides (affinity means: FH 220.4 nM ± 155.4 *versus* FHR1 62.4 nM ± 34.6, ANOVA, p-value = 0.022).

FHR1 Peptides	Binding core register	Position	Affinity(nM)	BindingLevel
LRWTAKQKLY**L**RTGE	WTAKQKLY**L**	2	20.57	SB
RWTAKQKLY**L**RTGES	WTAKQKLY**L**	1	29.86	SB
WTAKQKLY**L**RTGES**A**	AKQKLY**L**RT	0	44.76	SB
TAKQKLY**L**RTGES**A**E	LY**L**RTGES**A**	1	75.28	WB
AKQKLY**L**RTGES**A**EF	LY**L**RTGES**A**	4	72.63	WB
KQKLY**L**RTGES**A**EFV	LY**L**RTGES**A**	3	69.99	WB
QKLY**L**RTGES**A**EFVC	LY**L**RTGES**A**	2	123.7	WB

Table 6-22: Binding prediction of MHC class II DRB1*11:04 molecule for C-terminus FHR1-derived peptides (15 mer) determined by NetMHCIIpan 3.0 available online.

The affinity (nM) represents the IC50 values obtained during the analysis. SB = strong binder, WB = weak binder.

6.4 Discussion

In this chapter I focus on the association study between MHC class I and II alleles and the development of anti-FH in aHUS. I used as controls, a group of healthy subjects (57 Italians, 3 Jews, and 4 Iranians) carrying the homozygous deletion of *CFHR1* that is the main known susceptibility factor for the anti-FH development.[107] "Super controls" represent an ideal reference in the association studies. Indeed, I expect that in super controls other risk factors, in addition to the FHR1 deficiency, will be underrepresented, since the combination of more risk factors increases the probability to develop the disease and reduces the probability to belong to the healthy control group. On the contrary, the group of patients should be enriched for risk factors that contribute to the development of the disease. Here, I identify an association with *DRB1*11:04* allele: 38% of aHUS patients *versus* 13% of healthy controls are carriers of *DRB1*11:04* allele. Despite the threshold of statistical significance is not reached after the Bonferroni correction using the 64 super controls, I find that the *DRB1*11:04* allele is over-represented in our aHUS patients compared to several control populations reported on www.allele.frequencies.net and thus I cannot exclude at all the involvement of this *HLA* allele as a predisposing factor for anti-FH development.

These findings suggest a major role of *DRB1*11:04* allele in the pathogenesis of anti-FHs in aHUS patients although the low number of cases constitutes a limit for the analyses and further studies in other aHUS cohorts should confirm this data.

Anti-FHs are strongly associated to the presence of FHR1 deficiency that has a frequency about 5% in the European population and together with the *HLA-*

*DRB1*11:04* allele could increase the autoimmunity risk toward FH. In most autoimmune diseases multiple genetic polymorphisms contribute to the pathogenesis and each variant gives a small contribution to the disease.[151] *DRB1* alleles have received great attention in the association studies in the research field of autoimmune diseases mainly because MHC class II molecules are involved in the activation CD4+ T cells that regulate humoral immune responses to protein antigens. Previously reports have shown that *HLA-DRB1*11:04* allele was strongly associated with autoimmune diseases such as systemic sclerosis and systemic juvenile idiopathic arthritis.[187, 188] Most studies reported HLA typing in 2 digit resolution and *HLA-DRB1*11* has been found significantly over-represented in sarcoidosis patients compared to healthy controls and in association with idiopathic thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy like aHUS caused by anti-ADAMTS13 antibodies.[189-192] Interestingly, anti-FHs associated aHUS has been reported in a 17-years old Caucasian woman in association with anti-ADAMTS13 and affected by HUS/TTP.[131] Unfortunately, the HLA typing was not reported but the combination of anti-FHs and anti-ADAMTS13 suggest that there could be a unique predisposing factor to the autoimmunity and the *HLA-DRB1*11* is well known to be associated to anti-ADAMT13 and TTP.

A susceptibility factor in the immune system can trigger the disease in presence of specific predisposing variants in autoantigene coding genes. This "trigger-and-target" model is well described in Stanescu et al.[193] where the authors identified *HLA-DQA1* (the trigger) and PLA2R1 (the target) variants in association with idiopathic Membranous Nephropathy. In this paper the authors also reported the example of vitiligo, an autoimmune disease where predisposing *HLA* alleles in

combination with variants in autoantigen TYR gene, have been found. According with this model, in anti-FH mediated aHUS the "trigger" would be the presence of *HLA-DRB1*11:04* allele and "the target" the deficiency of FHR1 which is a FH autoantigen homologous protein and also a well known predisposing factor for autoantibodies against FH.

Finally, *in silico* analysis predict *DRB1*11:04* allele derived MHC molecules to bind more strongly C terminal FHR1 peptides than the homologous FH peptides which present a lower affinity. Interestingly, structure analyses of human and mouse autoimmune TCR-peptide-MHC complexes showed that the TCR binds self-peptide-MHC complex with an altered topology or with suboptimal anchors compared to foreign antigens bound to the same MHC molecules.[160, 161] I hypothesize that a weak affinity between FH peptides and MHC DRB1*11:04 molecules can determine a stability impairment of TCR-FH peptide-MHC complex that can lead to the loss of tolerance toward FH antigen. The tolerance could be guaranteed in individuals with FHR1 where auto-reactive CD4+ T cells can be eliminated during the negative selection in thymus.

In conclusion, in this chapter of my thesis, I report *HLA-DRB1*11:04* allele as a possible predisposing candidate genetic variant for anti-FH mediated aHUS development. Further works will be necessary to confirm this finding and to explore the presence of other genetic susceptibility factors that alone or in combination with this *HLA-DRB1*11:04* allele and the FHR1 deficiency can increase the risk for anti-FH development in aHUS.

7 Conclusions

In this thesis genetic and clinical features of 30 patients with anti-FH-associated aHUS have been described.

An accurate ELISA method has allowed to detect anti-FH antibodies and to reduce the percentage of false positive samples, leading to estimate that the prevalence of anti-FHs in our patient cohort is 9.8%.

The FHR1 deficiency was strongly associated with the development of anti-FHs and together with the age of disease onset between 4 and 12 years, it can predict the presence of anti-FHs in a patient with a diagnosis of aHUS. Interestingly, the FHR1 deficiency was also observed in the only healthy control resulted positive for anti-FHs suggesting that the lack of FHR1 can be also a risk factor for the development of autoantibodies in non affected subjects.

The protein domains recognized by anti-FHs were located at FH C terminus that is highly similar to FHR1. This observation suggests that the FHR1 is the crucial protein involved in the mechanism to maintain the central tolerance against FH and that its lack can predispose to autoimmunity.

Moreover, the presence of infectious prodromal signs and an age at the disease onset around 8 years indicate that common infections may trigger the development of autoantibodies in subjects with at risk genetic background.

Some pathogens are known to produce a number of RCA-like proteins with typical SCR domain structure that mimic human complement regulators, to evade the host immune

system.[46] I would hypothesize that if a pathogen producing a FHR1-like protein infects subjects with FHR1 deficiency, through a protein mimicry mechanism, the microbial protein could favor the activation of self reactive T cells which have escaped the thymus negative selection. T clones reactive against FHR1 are preferentially generated in FHR1 deficient subjects also carrying predisposing HLA alleles.

Here, the *HLA-DRB1*11:04* allele was found to be abundant in aHUS patients with anti-FHs compared to healthy controls carrying the FHR1 deficiency, despite this finding has to be confirmed in other cohorts of patients. The association between *HLA-DRB1*11:04* and the development of anti-FHs in aHUS could explain why the prevalence of anti-FH-associated aHUS is less than the frequency of the homozygous *CFHR1* deletion in healthy population. Indeed, the presence of both risk factors, the *HLA-DRB1*11:04* allele and the FHR1 deficiency, in combination with environmental triggers (infections) is required to determine a susceptibility state for the development of anti-FHs. Of note, the presence of both the *HLA-DRB1*11:04* allele and the FHR1 deficiency are not sufficient to determine the development of anti-FHs because some controls show both risk factors and do not have the disease. In view of the very low prevalence of the anti-FH associated aHUS (1:1,000,000), it is expected that other rare genetic variants or a rare combination of common variants are involved in the pathogenesis.

Thus, the presence of mutations in complement regulators (such as FH, MCP, FI), in THBD, and in activators of the AP of complement (such as C3 and FB) have been evaluated in our patients. A consistent percentage of patients are carriers of likely pathogenetic variants which can induce a complement activation that is *per se* a strong

stimulus for the adaptive immune system and can lead to the proliferation of the self reactive T clones and determine the onset of anti-FHs and of an autoimmune phenotype.

Moreover, it is well known that aHUS is a low penetrance disease and that multiple genetic risk factors, including mutations and at risk polymorphisms and haplotypes, are needed for the disease to manifest, at least in some individuals.

Interestingly, in our patients with anti-FHs, the polymorphic variants in complement genes, that have been documented as risk factors in patients with aHUS carrying mutations, do not seem to contribute to the disease. This was documented by comparing patients with, super controls, which are unaffected subjects carrying the major risk factor for this disease (*i.e.* the deficiency of FHR1). To the best of my knowledge, super controls have never been used in studies on anti-FH-associated aHUS and could represent a more effective approach in the association analysis since aHUS is a complex genetic disease despite its rarity. From this study it emerges that super controls shared with the patients the same *CFH* common variants and thus *CFH* seems not to be involved in the genetic predisposition to develop anti-FH-associated aHUS. The *CFH* promoter was also analyzed considering that maintaining self-tolerance may depend on FH levels, especially in the thymus. The absence of any associations with promoter variants has excluded the contribution of the *CFH* promoter to the disease. However, I cannot exclude that distal regions of the promoter, introns or intergenic regions can regulate FH levels in the thymus and determine a predisposition to autoimmunity.

Finally, common variants present in the other complement genes, which are already reported in association with complement mediated disease or with and increased complement activity, appear not to be involved as well.

In conclusion, here I have documented that the anti-FH-associated aHUS is a rare complex disease in which genetic and environmental factors are both involved. Anti-FH-mediated aHUS can represent a prototype of autoimmune disease in which antibodies are directed against a target protein for which a genetic defect has been found and a specific HLA allele predisposes to autoimmune response.

In future studies whole exome sequencing of patients and controls will be done to identify other genetic factors implicated in the pathogenesis of anti-FHs focusing on the genomic loci known to be involved in autoimmunity. Super controls will be used as reference to evaluate the presence of predisposing variants in aHUS patients which could increase the OR for the disease and/or the presence of protective variants in super controls that do not manifest the disease although carrying the major risk for anti-FH development.

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